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(54) Title: ANALOGS OF FIBROBLAST GROWTH FACTOR

(57) Abstract

Disclosed are recombinant basic fibroplast growth factor analogs possessing part or all of the primary structural conformation and one or more of the biological properties of a mammalian (e.g., human) basic fibroblast growth factor, wherein at least one of the cysteine residues of the naturally occurring basic fibroblast growth factor is replaced with a residue of a different amino acid. Also disclosed is a process for producing such analogs wherein a host cell is transformed or transfected with an exogenous DNA sequence encoding for the basic fibroblast growth factor analogs. Purification methods for the analogs are also disclosed as well as *in vivo* applications. Biologically active analogs of basic fibroplast growth factor are more stable and facilitate purification of basic fibroplast growth factor.

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ANALOGS OF FIBROBLAST GROWTH FACTOR

The present invention relates to analogs of basic
fibroblast growth factor. In particular, the present
5   invention refers to analogs of recombinant basic
fibroblast growth factor ("r-bFGF") in an _E. coli_
(Escherichia coli) host strain and to polynucleotides
encoding such factors. The following nomenclature is
utilized: FGF = fibroblast growth factor; acidic
10   FGF = aFGF; naturally occuring or natural FGF = n-FGF;
human FGF = h-FGF; and bovine FGF = b-FGF; human basic
FGF = h-bFGF; bovine basic FGF = b-bFGF; recombinant
basic FGF = r-bFGF; recombinant human basic FGF =
rh-bFGF; and recombinant bovine basic FGF = rb-bFGF.
15

BACKGROUND

Fibroblast Growth Factor (FGF) was first described
by Gospodarowicz, Nature, 249, 123 (1974) as an activity
20   derived from bovine brain or pituitary tissue which was
mitogenic for fibroblasts and endothelial cells. It was
later noted that the primary mitogen from brain was
different than that isolated from pituitary. These two
factors were named acidic and basic FGF because they had
25   similar if not identical biological activities but
differed in their isoelectric points. Subsequently
other endothelial cell mitogens were isolated from a
number of tissues and tumors which are very similar or
identical to basic FGF. Such factors include, for
30   example, hepatoma-derived growth factor (Klagsbrun, et
al, PNAS, 83, 2448-2452 (1986) and Lobb et al,
J.Biol.Chem., 23, 6295-6299 (1984)), chondrosarcoma-
derived growth factor (Shing et al, Science, 223, 1296-
1299 (1984)), beta retina-derived growth factor (Baird
35   et al, Biochemistry, 24, 7855-7860 (1985)), cartilage-
derived growth factor (Sullivan and Klagsbrun,

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J. Biol. Chem., 260, 2399-2403 (1985)), astroglial
growth factor 2 (Pettman et al, FEBS Lett., 189, 102-
108), eye-derived growth factor (Courty et al,
Biochimie, 67, 265-2698 (1985)), cationic hypothalamus-
5   derived growth factor (Klagsbrun and Shing, PNAS, 82,
805-809 (1985)), class 2 and beta heparin-binding growth
factors (Lobb and Fett, Biochemistry, 23, 6265-6299
(1984); Lobb et al, Biochem., 24, 4969-4973 (1985);
Lobb et al, BBRC, 131, 586-592 (1985); Lobb et al,
10 J.Biol.Chem., 261, 1924-1928 (1986)), and a component of
macrophage-derived growth factor (Baird et al, BBRC,
126, 358-364 (1985)). All of the above factors share
bFGF's property of binding tightly to heparin and all
are basic proteins. A similar group of heparin-binding
15 factors, typified by aFGF, have also been found. These
molecules elute from heparin at a lower sodium chloride
concentration and have acidic isoelectric points. The
heparin binding property of these factors has
facilitated their purification, allowing isolation of
20 sufficient protein for amino acid sequence analysis in
several cases. Although the use of heparin has
facilitated purification of FGF, the use of heparin in
large scale purification procedures is undesirable
because of the expense, possible contamination of
25 product with heparin, and loss of yield due to
irreversible binding to heparin. Acidic and basic FGF
are probably derived from the same ancestral gene and
are 55% homologous in amino acid sequence and have the
same intron/exon structure. Southern blotting
30 experiments suggest that there is only one gene each for
acidic and basic FGF; differences between the molecules
isolated from different tissues are probably due to
post-translational processing. The range of biological
activities of the two classes appears to be identical,
35 although bFGF is about ten times more potent than aFGF
in most bioassay systems.

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Basic FGF is a single chain, non-glycosylated
protein having a molecular weight of approximately
16,500. Basic FGF contains four cysteine residues, but
the number of disulfide bonds, if any, is unknown. A
5 primary translation product having 155 amino acids has
been proposed for bFGF, but the major form found in
pituitary tissue has 146 amino acids. Several molecular
weight forms, differing in length at the N-terminus,
have been isolated from different tissues, all of which
10 appear to retain biological activity. Basic FGF is an
extremely basic protein, with an isoelectric point of
9.6. Basic FGF binds avidly to heparin, eluting from
heparin sepharose columns at around 1.6 M NaCl. The
biological activity of bFGF is destroyed by heat (70°C)
15 or by detergents. In the genome, coding sequences for
this translation product are interrupted by two introns;
the first splits codon 60 and the second separates
codons 94 and 95. The size of the entire genomic coding
region is not known, but it is at least 34 kb in
20 length. The gene for bFGF is located on chromosome 4.
The first sequence data for bFGF was published by
Bohlen et al PNAS, 81, 5364-5368, (1984) who determined
the N-terminal 15 amino acids of material purified from
bovine pituitary tissue. Subsequently, Esch et. al.,
25 PNAS, 82, 6507-6511, (1985) reported the complete
sequence of bFGF from bovine pituitary and at the same
time compared it with amino terminal sequence from
aFGF. PCT patent application WO 86/07595 discloses the
production of b-bFGF in E. coli. However, the reported
30 yields of product are extremely low. Cloning of the
gene for b-bFGF was first reported by Abraham et al
Science, 233, 545-548, and a later paper by the same
authors described the nucleotide sequence and genomic
organization of h-bFGF (EMBO Journal, 5, 2523-2528
35 (1986)). Bovine and human bFGF are known to differ only
by two amino acids.

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Although highly purified preparations of bFGF have
only recently been available for testing, many in vitro
studies have been published using material of various
states of purity. In these studies, bFGF has been shown
5 to be a potent mitogen for a wide variety of cells of
mesodermal origin and may be chemotactic for endothelial
cells and fibroblasts. In addition, naturally occurring
and tissue derived r-bFGF appears to induce
neovascularization in both the rabbit cornea and chick
10 chorioallantoic membrane assays, thus bFGF may be useful
in accelerating the healing of wounds. Fourtanier
et al, J.Inv.Derm., 87, 76-80 (1986) disclosed that a
preparation derived from bovine retina was able to
stimulate neovascularization and reepithelialization and
15 to promote the healing of wounds in a guinea pig blister
model. Davidson et al, J.Cell.Biol., 100, 1219-1227
(1985) have shown accelerated wound repair accompanied
by increased granulation tissue and collagen
accumulation to be induced by a bovine cartilage derived
20 factor in a rat wound model system. Buntrock and
coworkers, Exp.Path., 21, 46-53, and Exp.Path., 21,
62-67 (1982) have also reported increases in granulation
tissue and neovascularization along with accelerated
healing of wounds in rats using an extract of bovine
25 brain tissue.

SUMMARY OF THE INVENTION

The present invention relates to analogs of bFGF
30 that are more stable than the naturally occurring bFGF
and facilitate purification of r-bFGF. An analog of
bFGF having an amino acid sequence comprising cysteine
residues is provided, wherein at least one, and more
preferably two, of said cysteine residues is replaced by
35 a different amino acid residue.

The present invention also relates to a preferred
process for producing bFGF analogs, said process
comprising:
1) growing under suitable nutrient conditions,
5   E. coli host cells transformed with a DNA plasmid vector
wherein the DNA plasmid vector comprises a DNA sequence
coding for E. coli host expression of a bFGF analog
having part or all of the primary structural
conformation of bFGF and one or more of the biological
10 properties of human basic fibroblast growth factor
wherein at least one cysteine residue is replaced by a
residue of a different amino acid (hereinafter referred
to as "bFGF analogs"), a regulated promoter sequence,
and a temperature inducible copy number control gene;
15   2) isolating desired bFGF analogs of the
expression of DNA sequences in said vector; and
3) purifying the desired bFGF analogs.
The present invention further relates methods for
purifying E. coli derived r-bFGF analogs using non-
20 heparin containing chromatographic systems.
DNA sequences coding for all or part of rh-bFGF
analogs are provided. Such sequences preferably may
include: (1) the incorporation of codons "preferred" for
expression by selected E. coli host strains ("E. coli
25 expression codons"); (2) the provision of sites of
cleavage by restriction endonuclease enzymes; and/or,
(3) the provision of additional initial, terminal or
intermediate DNA sequences which facilitate construction
of readily expressed vectors. The novel DNA sequences
30 of the invention include sequences useful in securing
expression in E. coli host cells of bFGF analogs.
DNA sequences of the invention are specifically
seen to comprise: (a) the DNA sequences set forth in
Fig. 2 wherein at least one codon encoding a cysteine
35 residue is replaced by a codon encoding a different
amino acid residue (hereinafter "analog sequences");

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(b) a DNA sequence which hybridizes to one of the analog
sequences or to fragments thereof; and (c) a DNA
sequence which, but for the degeneracy of the genetic
code, would hybridize to one of the analog sequences.

5   Specifically comprehended by part (c) are manufactured
DNA sequences encoding bFGF analogs which DNA sequences
may incorporate codons facilitating translation of
messenger RNA in the preferred microbial hosts. Such
manufactured sequences may readily be constructed

10   according to the methods of Alton, et. al., PCT
published application WO 83/04053.

Purified and isolated bFGF analogs having one or
more of the biological properties (e.g., immunological
properties and in vitro biological activity) and

15   physical properties (e.g., molecular weight) of
naturally-occurring bFGF including allelic variants
thereof are described. These bFGF analogs may also be
characterized by being the product of the preferred
E. coli host expression of exogenous DNA sequences. The

20   bFGF analogs of the present invention which are
expressed from the preferred E. coli host cells may
include an initial methionine amino acid residue (at
position 1 as shown in Fig. 2). Alternatively, one or
more of the terminal amino acid residues may be deleted

25   from the DNA sequence while substantially retaining the
biological activity of naturally occurring bFGF, as is
known to those skilled in the art.

Various replicable cloning vehicles, expression
vehicles and transformed E. coli cultures, all harboring

30   the altered genetic information necessary to effect the
production of E. coli derived bFGF analogs are also
contemplated within the scope of the present invention.

Site-directed mutagenesis may be used to convert
the gene for b-bFGF into one coding for rh-bFGF.

35   Additionally, the bovine gene and human gene can be
modified by site-directed mutagenesis to convert at
least one of the cysteine residues.

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Numerous aspects and advantages of the invention
will be apparent to those skilled in the art upon
consideration of the following detailed description
which provides illustrations of the practice of the
5 invention in its presently preferred embodiments.

Brief Description of the Drawings

Fig. 1 is a diagrammatic representation of the bFGF
10 gene assembly and cloning.

Fig. 2 is the nucleotide and amino acid sequences
of r-bFGFs. The solid boxes outline the nucleotide and
resultant amino acid changes which were produced by
site-directed mutagenesis in order to convert the bovine
15 gene to one coding for rh-bFGF. The dashed line boxes
highlight the changes made to convert the bFGF gene into
one coding for the bFGF analogs of the present
invention.

Fig. 3 is a graph of the mitogenic activity of
20 r-bFGFs on NIH3T3 cells. The mitogenic effect of
rh-bFGF (•) and rb-bFGF (o) and recombinant human
(ser-70,88) bFGF (x) on NIH3T3 cells is shown. The dose
of r-bFGF is plotted against the percentage of maximal
stimulation of DNA synthesis as measured by ^{3}H thymidine
25 uptake at that dose.

Fig. 4 is a photograph of a silver stained gel from
sodium dodecyl sulfate polyacrylamide gel
electrophoresis ("SDS-PAGE") of purified r-bFGFs.

Fig. 5 is a high pressure liquid chromatography
30 (HPLC) profile of trypsin-digested native (top panel)
and S-carboxymethylated (bottom panel) rb-bFGF. Arrows
indicate the peptides which show major differences in
the retention time upon S-carboxymethylation.

Fig. 6 is a circular dichroic spectrum of rb-bFGF
35 in the far (left) and near (right) UV regions.

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Fig. 7 is a graph of the mitogenic activity of the Ser-26,70,88,93 bFGF analog on NIH3T3 cells. The dose of r-bFGF is plotted against the percentage of maximal stimulation of DNA synthesis as measured by ^{3}H-thymidine uptake at that dose.

Fig. 8 is a diagram for wounding and observation in the rabbit ear in vivo study of Example 16.

DETAILED DESCRIPTION

Novel bFGF analogs are provided in accordance with the present invention, wherein at least one, and more preferably two, of the cysteines found in natural bFGF are replaced with a different amino acid residue. These analogs have been found to exhibit a surprisingly marked increase in stability over the natural bFGF. It is believed that the more stable bFGF analogs of the present invention may also increase the efficacy of bFGF in wound healing treatments and in surgery.

Also provided by the present invention are manufactured or synthetic genes capable of directing synthesis, in selected microbial hosts (e.g., bacteria, yeast, and mammalian cells in culture), of the bFGF analogs.

Further comprehended by the present invention are pharmaceutical compositions comprising effective amounts of bFGF analogs of the invention together with suitable diluents, adjuvants and/or carriers useful in wound healing and surgical applications, including, but not limited to, the healing of surface wounds, bone healing, angiogenesis (formation of blood vessels, especially important in the healing of deep wounds), nerve regeneration, and organ generation and regeneration.

As used herein, the term "tissue-derived basic fibroblast growth factor" refers to basic fibroblast growth factor derived from tumors, eucaroytic cells maintained in culture, normal tissues and the like.

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As employed herein, the term "manufactured" as applied to a DNA sequence or gene shall designate a product either totally chemically synthesized by assembly of nucleotide bases or derived from the

5   biological replication of a product thus chemically synthesized. As such, the term is exclusive of products synthesized by cDNA methods of genomic cloning methodologies which involve starting materials which are initially of biological origin.

10   As employed herein, the term "synthesized" refers to site-directed mutagenesis or other alteration of a previously manufactured gene.

As herein described, the term "cysteine residues existing as free sulfhydryls" refers to cysteine

15   residues that are not involved in forming disulfide bonds.

The E. coli derived recombinant bFGF was produced in accordance with the following general procedure:

The amino acid sequence of b-bFGF published by Esch

20   et al PNAS, 82, 6507-6511 (1985) was used as a basis for manufacturing a bFGF gene for expression in E. coli. The nucleotide sequence of this manufactured gene includes codons most often used by E. coli and convenient restriction sites to be used for cloning

25   purposes. Illustrative of a manufactured gene is the gene represented in Table I. Table I represents a manufactured gene for b-bFGF, while Table II represents a synthetic gene for h-bFGF. The nucleotide sequence of the manufactured gene for b-bFGF and the amino acid

30   sequence from which it was derived is shown in Figure 2. The solid boxes outline the nucleotide and resultant amino acid changes which were produced by site-directed mutagenesis in order to convert the manufactured bovine gene to a synthetic gene coding for

35   h-bFGF. The dashed line boxes highlight the subsequent changes made to convert the synthetic h-bFGF gene into

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analog sequences wherein one or more of the cysteine
·residues are replaced with serine residues. It is
recognized that site-directed mutagenesis can be used to
generate other b-bFGF analogs. In other words, other
5   amino acids can be used to replace the cysteine
residues. Generally, the amino acid selected to replace
the cysteine residue is selected on the basis of its
ability to create an analog which would retain a
similiar structure but avoid dimer formation. Examples
10   of such amino acids include serine, alanine, aspartic
acid and asparagine. Other amino acids which may be
substituted for cysteine will be apparent to those
skilled in the art.

       Oligonucleotides corresponding to both strands of
15   the b-FGF gene were manufactured in overlapping sections
and assembled into two larger sections by hybridization
and subsequent ligation. The two larger sections were
then cloned into an appropriate phage vector (i.e.,
M13mp18) for nucleotide sequence analysis. Such phage
20   vectors are readily ascertained by one of ordinary skill
in the art. Upon verification of the correct sequence,
both sections were excised by restriction endonuclease
digestion, gel isolated, and ligated into an appropriate
expression vector. Expression of the bFGF gene encoded
25   on the expression vector is regulated by a regulated
promoter sequence and the temperature inducible copy
number control genes located on the expression vector.
The term "regulated promoters" as used herein, refers to
P_L promoters (e.g., promoters derived from a λ phage)
30   and foreshortened P_L promoter. Expression vectors
containing such regulated promoters and temperature
inducible copy number control genes are described in
European Patent Application No. 136,490. Growth of the
expression vector containing the bFGF gene in an
35   appropriate E. coli host strain yielded rb-bFGF. When
the rb-bFGF-containing cells were lysed and subjected to

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TABLE I

Bovine basic Fibroblast Growth Factor/manufactured gene

```
        10                    30                    50
CTAGAAGGAGGAATAACATATGCCAGCTCTGCCAGAAGATGGTGGATCCGGTGCTTTCCC
GATCTTCCTCCTTATTGTATACGGTCGAGACGGTCTTCTACCACCTAGGCCACGAAAGGG


        70                    90                   110
GCCAGGTCATTTCAAAGATCCGAAACGTCTGTACTGCAAAAACGGTGGTTTTTTCCTGCG
CGGTCCAGTAAAGTTTCTAGGCTTTGCAGACATGACGTTTTTGCCACCAAAAAAGGACGC


       130                   150                   170
TATCCATCCGGATGGTCGTGTTGATGGTGTACGTGAGAAATCTGATCCGCATATCAAACT
ATAGGTAGGCCTACCAGCACAACTACCACATGCACTCTTTAGACTAGGCGTATAGTTTGA


       190                   210                   230
GCAGCTGCAAGCTGAAGAGCGTGGTGTAGTTTCTATTAAAGGTGTATGTGCTAACCGGTA
CGTCGACGTTCGACTTCTCGCACCACATCAAAGATAATTTCCACATACACGATTGGCCAT


       250                   270                   290
CCTGGCTATGAAAGAAGACGGTCGTCTGCTGGCTTCTAAGTGTGTTACTGACGAATGTTT
GGACCGATACTTTCTTCTGCCAGCAGACGACCGAAGATTCACACAATGACTGCTTACAAA


       310                   330                   350
CTTTTTCGAACGTCTGGAATCTAACAACTACAACACTTACAGATCTCGTAAATACTCTTC
GAAAAAGCTTGCAGACCTTAGATTGTTGATGTTGTGAATGTCTAGAGCATTTATGAGAAG


       370                   390                   410
CTGGTATGTAGCTCTGAAACGTACTGGTCAGTACAAACTGGGTCCGAAGACTGGTCCGGG
GACCATACATCGAGACTTTGCATGACCAGTCATGTTTGACCCAGGCTTCTGACCAGGCCC


       430                   450                   470
TCAGAAAGCTATCCTGTTTCTGCCGATGTCTGCTAAATCTTAATAGCTCGAGAAGCTT
AGTCTTTCGATAGGACAAAGACGGCTACAGACGATTTAGAATTATCGAGCTCTTCGAA
```

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TABLE II

Human basic Fibroblast Growth Factor/synthetic gene

```
        10                    30                    50
CTAGAAGGAGGAATAACATATGCCAGCTCTGCCAGAAGATGGTGGATCCGGTGCTTTCCC
GATCTTCCTCCTTATTGTATACGGTCGAGACGGTCTTCTACCACCTAGGCCACGAAAGGG


        70                    90                    110
GCCAGGTCATTTCAAAGATCCGAAACGTCTGTACTGCAAAAACGGTGGTTTTTTCCTGCG
CGGTCCAGTAAAGTTTCTAGGCTTTGCAGACATGACGTTTTTGCCACCAAAAAAGGACGC


        130                    150                    170
TATCCATCCGGATGGTCGTGTTGATGGTGTACGTGAGAAATCTGATCCGCATATCAAACT
ATAGGTAGGCCTACCAGCACAACTACCACATGCACTCTTTAGACTAGGCGTATAGTTTGA


        190                    210                    230
GCAGCTGCAAGCTGAAGAGCGTGGTGTAGTTTCTATTAAAGGTGTATGTGCTAACCGGTA
CGTCGACGTTCGACTTCTCGCACCACATCAAAGATAATTTCCACATACACGATTGGCCAT


        250                    270                    290
CCTGGCTATGAAAGAAGACGGTCGTCTGCTGGCTTCTAAGTGTGTTACTGACGAATGTTT
GGACCGATACTTTCTTCTGCCAGCAGACGACCGAAGATCACACAATGACTGCTTACAAA


        310                    330                    350
CTTTTTCGAACGTCTGGAATCTAACAACTACAACACTTACAGATCTCGTAAATACACTTC
GAAAAAGCTTGCAGACCTTAGATTGTTGATGTTGTGAATGTCTAGAGCATTTATGTGAAG


        370                    390                    410
CTGGTATGTAGCTCTGAAACGTACTGGTCAGTACAAACTGGGTTCGAAGACTGGTCCGGG
GACCATACATCGAGACTTTGCATGACCAGTCATGTTTGACCCAAGCTTCTGACCAGGCCC


        430                    450                    470
TCAGAAAGCTATCCTGTTTCTGCCGATGTCTGCTAAATCTTAATAGCTCGAGAAGCTT
AGTCTTTCGATAGGACAAAGACGGCTACAGACGATTTAGAATTATCGAGCTCTTCGAA
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low speed centrifugation, about 30% to about 70% of
rb-bFGF was found in soluble form in the supernatant
fraction. Purification using non-heparin containing
chromatographic systems, i.e., affinity chromatography

5   resulted in a polypeptide product which was estimated to
be at least 95% pure by polyacrylamide gel
electrophoresis and contained virtually no endotoxin or
DNA contamination.

    Site-directed mutagenesis was employed to convert

10   the bovine gene into one coding for human bFGF. The
same purification scheme used for rb-bFGF was also used
to purify rh-bFGF.

    The mitogenic activity of the E. coli derived
rb-bFGF, rh-bFGF, and the bFGF analogs of the present

15   invention was measured using an in vitro mitogenic assay
based on the increase in radiolabeled thymidine uptake
by mouse 3T3 cells which accompanies increased DNA
synthesis during cell division.

    Oligonucleotide site-directed mutagenesis was used

20   to modify the rh-bFGF gene so that sequences which coded
for some or all of the cysteine residues would now
encode for different amino acid residues. The bFGF
analogs of the present invention were surprisingly found
to impart markedly greater stability to the bFGF

25   molecule, while not detracting from bFGF activity.

    The following examples serve to further illustrate
the embodiments of the present invention. The term
"OD", as used in these examples, refers to optical
density units at 600 nm.

30

EXAMPLE 1

Preparation of a Manufactured Gene Encoding b-bFGF

35   This example relates to the preparation of a
manufactured gene encoding b-bFGF wherein E. coli

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expression preference codons are included. The protocol
employed to prepare the manufactured gene encoding a
b-bFGF product is generally described in the disclosure
of Alton, et al,, PCT Publication No. WO83/04053 which is
5   incorporated by reference herein. The genes were
designed for initial assembly of component
oligonucleotides into multiple duplexes which, in turn,
were assembled into 2 discrete sections. These sections
were designed for ready amplification and, upon removal
10 from the amplification system, could be assembled
sequentially or through a multiple fragment ligation in
a suitable expression vector.

Assembly of Section I of Fibroblast Growth Factor
15

     200 pm of each of the 16 oligomers required for
assembly of section I represented in Table III were
measured into eppendorf tubes and dried on a speed
vacuum pump. 320 µl of a kinase mix was prepared which
20 contained 32 µl of 10x ligation buffer (50 M HEPES, pH
7.6), 0.7 µl of 10 mM adensosine triphosphate (ATP),
1 µl of $3x10^7$ counts/minute/µl of radiolabelled ATP, and
266 µl of water. The reagents were combined in a tube
of kinase (Boehringer Mannheim, Ingelheim, West Germany)
25 which contained 20 µl of the kinase enzyme at a
concentration of 10 units/µl. This kinase mix was
aliquoted into each of tubes 2-15 containing
oligonucleotides 2-15, respectively. Tubes containing
oligonucleotides 1 and 16 received ligation buffer
30 only. Tubes containing 2-15 were incubated at 37°C for
45 minutes. At the end of that time period, 1/4 µl
aliquots from each tube were spotted onto DE81 paper
chromatography strips (Whatman, Maidstone, U.K.) eluted
with 0.35 M ammonium formate and analyzed on a liquid
35 scintillation counter. Liquid scintillation analysis
showed that more than 1/2 of the counts were at the

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TABLE III

FGF OLIGOMERS, SECTION I

1) 5′ CTAGAAGGAGGAATAACATATGCCAGCTCT 3′

2) 5′ GCCAGAAGATGGTGGATCCGGTGCTTTCCC 3′

3) 5′ GCCAGGTCATTTCAAAGATCCGAAACGTCTG 3′

4) 5′ TACTGCAAAAACGGTGGTTTTTTCCTGCGTA 3′

5) 5′ TCCATCCGGATGGTCGTGTTGATGGTGTAC 3′

6) 5′ GTGAGAAATCTGATCCGCATATCAAACTGCA 3′

7) 5′ GCTGCAAGCTGAAGAGCGTGGTGTAGTTT 3′

8) 5′ CTATTAAAGGTGTATGTGCTAACCGGTACCTG 3′

9) 5′ CTGGCAGAGCTGGCATATGTTATTCCTCCTT 3′

10) 5′ TGGCGGGAAAGCACCGGATCCACCATCTT 3′

11) 5′ AGTACAGACGTTTCGGATCTTTGAAATGACC 3′

12) 5′ ATGGATACGCAGGAAAAAACCACCGTTTTTGC 3′

13) 5′ TCACGTACACCATCAACACGACCATCCGG 3′

14) 5′ CAGCTGCAGTTTGATATGCGGATCAGATTTC 3′

15) 5′ ATAGAAACTACACCACGCTCTTCAGCTTG 3′

16) 5′ AATTCAGGTACCGGTTAGCACATACACCTTTA 3′

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origin. As a result, 2 μl of 10 mM ATP were added to
each of the tubes containing 2-15 oligonucleotides, and
the tubes incubated an additional 45 minutes at 37°C.
At the end of this time period, all tubes were boiled
5   for 10 minutes then centrifuged and combined into
duplexes. This was done by adding the contents of tube
9 to tube 1 (duplex #1), tube 10 to tube 2 (duplex #2),
tube 11 to tube 3 (duplex #3), tube 12 to tube 4 (duplex
#4), tube 13 to tube 5 (duplex #5), tube 14 to tube 6
10 (duplex #6), tube 15 to tube 7 (duplex #7), and tube 16
to tube 8 (duplex # 8). These eight mixtures of
oligonucleotides were then boiled and slow cooled to
room temperature to allow formation of the duplexes.
The duplexes were then combined so that duplexes #1 and
15 #2 were combined (tetramer 1+2), duplexes #3 and #4
(tetramer 3+4) were combined, duplexes #5 and #6
(tetramer 5+6) were combined, and finally duplexes #7
and #8 (tetramer 7+8) were combined. To each of these
tubes, now containing tetramers, 2 μl of 10 mM ATP and
20 2 μl of T4 DNA ligase from Boehringer Mannheim were
added. These ligation mixtures were then incubated for
10 minutes at 37°C and then at room temperature for
1 hour. At this point, the tetrameric mixtures were
pooled again so that the tetramers 1+2 and 3+4 were
25 combined together and tetramers 5+6 and 7+8 were
combined together. To each of the two resulting
ligation mixtures was added an additional 2 μl of 10 mM
ATP and 2 μl of T4 DNA ligase. The mixtures were
incubated for 10 minutes at 37°C and then at room
30 temperature for 1 hour. Finally the entire ligation was
pooled together, that is, the contents of both tubes
were added together, an additional 8 μl of ligase were
added to the ligation mixture, and the entire mixture
incubated for 10 minutes at 37°C and then overnight at
35 4°C. Following overnight ligation, one 10 μl aliquot
was analyzed on an 8% polyacrylamide gel made up with

7 M urea. A band could be discerned adjacent to the 242
base pair HpaII marker, which indicated that the
ligation was complete. An 8% polyacrylamide gel was
made which also contained 7 M urea. The ligation mix

5   was ethanol precipitated, dried and then taken up in
80 μl of 80% formamide. Half of this ligation mix was
then loaded onto the preparative gel adjacent to a lane
containing HpaII cut PBR322 markers. The gel was run
until the xylene cyanol dye marker had reached the

10   bottom of the gel. The gel was then removed from the
electrophoresis apparatus and placed in a film cassette
next to a piece of Kodak X-Ray film. The bands were
visualized by developing the film, and a gel slice just
above the HpaII 242 marker on the adjacent lane

15   removed. This slice contained the 244 base pair band
expected for the completely ligated section I of
fibroblast growth factor. This gel slice was extruded
through a syringe into an eppendorf tube, and covered
with Maxam Gilbert Gel Elution solution and incubated

20   overnight at 37°C. The contents of the tube were then
filtered through a glass fiber filter placed in a
syringe barrel and the supernatant was extracted three
times with N-butanol and ethanol precipitated. The
dried pellet was then taken up in 200 μl of a solution

25   of 10 mM Tris-HCl and 1 mM ethylene diamine tetraacetic
acid (EDTA), and reprecipitated with ethanol after
removing the polyacrylamide residue which was
centrifuged in the bottom of the tube. The ethanol
precipitated sample contained about 37,000 counts per

30   minute which corresponded to about 1.5 pm of duplex
based upon the radioactivity corresponding to the
oligomers required for this ligation. These 1.5 pm were
then dissolved in 20 μl of ligation buffer containing
3×10^7 counts per minute of radiolabelled ATP. A 1/4 μl

35   aliquot was removed and spotted on a DE81 strip. Then,
1 μl of kinase was added and the tube was incubated at

- 18 -

TABLE IV

FGF OLIGOMERS, SECTION II

17) 5'   AATTCGGTACCTGGCTATGAAAGAAGACGGTCGTCTGCTGG 3'

18) 5'   CTTCTAAGTGTGTTACTGACGAATGTTTCTTTTTCGAACG 3'

19) 5'   TCTGGAATCTAACAACTACAACACTTACAGATCTCGTAAA 3'

20) 5'   TACTCTTCCTGGTATGTAGCTCTGAAACGTACTGGTCAGT 3'

21) 5'   ACAAACTGGGTCCGAAGACTGGTCCGGGTCAGAAAGCTATCC 3'

22) 5'   TGTTTCTGCCGATGTCTGCTAAATCTTAATAGCTCGAGA 3'

23) 5'   GAAGCCAGCAGACGACCGTCTTCTTTCATAGCCAGGTACCG 3'

24) 5'   CAGACGTTCGAAAAAGAAACATTCGTCAGTAACACACTTA 3'

25) 5'   ATGATTTACGAGATCTGTAAGTGTTGTAGTTGTTAGATTC 3'

26) 5'   TTGTACTGACCAGTACGTTTCAGAGCTACATACCAGGAAG 3'

27) 5'   AAACAGGATAGCTTTCTGACCCGGACCAGTCTTCGGACCCAGT 3'

28) 5'   AGCTTCTCGAGCTATTAAGATTTAGCAGACATCGGCAG 3'

37°C for 45 minutes. At this point, 1/4 µl was removed
from the tube and spotted on a second DE81 strip. Both
strips were then eluted with 0.35 M ammonium formate and
then cut into sections and counted on a liquid
scintillation counter. The before and after strips
clearly showed that counts were incorporated at the
origin, therefore, the kination reaction was driven to
completion by the addition of with 1 µl of 10 mm ATP and

- 19 -

incubation for 30 minutes longer at 37°C. Then the
kination mixture was boiled for 5 minutes and slow
cooled to room temperature.

5   Assembly of Section II of Fibroblast Growth Factor

     Fibroblast Growth Factor Section II was assembled
     in a similar manner. 200 pm of each of the 12
     oligonucleotides represented in Table IV were measured
10   into eppendorf tubes and speed-vacuumed to dryness. The
     drying was repeated with 100 µl of 80% ethanol. A
     kinase mix was prepared which contained 24 µl of 10x
     ligation buffer, 2 µl of radiolabelled ATP (1.5×10^7
     counts/minute/µl), 0.5 µl of 10 mM ATP, 20 µl of kinase
15   and 193 µl of water giving the total volume of 240 µl in
     the kinase mix. 20 µl of this mixture were added to
     each of the tubes, containing oligonucleotides 18-27,
     respectively, to be kinased. Tubes containing
     oligonucleotides 17 and 28 were given ligation buffer
20   only. The tubes were then incubated for 45 minutes at
     37°C at which time a 1/4 µl aliquot was removed from
     each tube and spotted onto DE81 strips. The DE81 strips
     were then eluted with 0.35 M ammonium formate and
     checked with a liquid scintillation counter to determine
25   the number of counts at the origin. The analysis showed
     that the kination was proceeding. At this point, 1 µl
     of 10 mM ATP was added to each tube and the tubes were
     incubated for an additional 45 minutes at 37°C. The
     tubes were boiled for 5 minutes and then centrifuged and
30   combined to form duplexes. Oligonucleotide #23 was
     combined with #17 (duplex #17), #24 with #18 (duplex
     #18), #25 with #19 (duplex #19), #26 with #20 (duplex
     #20), #27 with #21 (duplex #21), and #28 with #22
     (duplex #22). These duplex mixtures were then boiled
35   and slow cooled to room temperature over a period of 1
     hour. The duplexes were then combined to form

- 20 -

tetramers. Duplexes 17+18 were combined to form
tetramer 17, duplexes 19+20 were combined to form
tetramer 19, and duplexes 21+22 were combined to form
tetramer 21. These were annealed at 37°C for 10
5 minutes. To each tetrameric mixture were added 2 μl of
10 mM ATP and 2 μl of T4 DNA ligase. The 3 ligations
were incubated overnight at 4°C. At this point 4 μl
aliquots were removed from each of the three tubes
containing the tetramers and run on a 10% polyacrylamide
10 gel made with 7 M urea. Autoradiography of the gel
showed that the ligation was proceeding. Tetramers 17
and 19 were pooled and 4 μl of ligase were added along
with 4 μl of 10 mM ATP. The 8 piece ligation was then
incubated at 37°C for 15 minutes and then at 4°C at 6
15 hours before adding the last tetramer to the ligation
mixture. At this point tetramer 21 was added to the
octameric ligation mixture and the entire ligation was
incubated at 37°C for 15 minutes. 5 μl of ligase and
5 μl of 10 mM ATP were added, and the resultant mixture
20 incubated at 37°C for 15 minutes. 5 μl of ligase and
5 μl of 10 mM ATP were again added to the ligation
mixture, and the entire ligation incubated overnight at
4°C. A check of the full ligation on an 8%
polyacrylamide gel made with 7 M urea showed a prominent
25 band at 242 base pairs as expected. The ligation mix
was phenol extracted and ethanol precipitated before
loading onto a prep gel. 1/2 of the ligation mix was
loaded onto an 8% 7 M urea gel and the 242 base pair
product was visualized by autoradiography, removed, and
30 purified as described for section I of the bFGF.

EXAMPLE 2

Cloning and Expression of rb-bFGF
35

The b-bFGF gene was synthesized in two sections as
described in Example 1. Each section was cloned into

- 21 -

M13mp18 for sequence verification before assembly into
an expression vector, pCFM1156. In preparation for the
cloning of Section I, M13mp18 was digested with a
threefold excess of restriction enzymes EcoRI and XbaI
5 for 2 hrs. The reaction was terminated by extraction
with an equal volume of phenol followed by extraction
with chloroform and precipitation with 2.5 volumes of
ethanol. The DNA pellet was washed with ethanol, dried
under vacuum and dissolved in 10 mM Tris, 0.1 mM EDTA,
10 pH 7.4. Ligation was carried out by incubation of 0.06
pmole of the M13mp18 vector prepared as described with
0.3 pmole of the synthetic FGF Section I in 50 mM tris
(pH 7.4), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM
spermidine, 1 mM ATP, 100 µg/ml bovine serum albumin
15 (BSA), and 1 unit T4 ligase for 4 hours at 14°C.
E. coli JM109 host cells were made competent by
centrifugation of cells from an exponentially growing
culture, suspension in ice-cold 50 mM $CaCl_2$ at a
concentration of 1.2 OD/ml for 20 minutes, followed by
20 recentrifugation and resuspension of the cells in the
same solution at a concentration of 12 OD/ml. Aliquots
of the ligation mixture (0.1-10 µl) were added to 200 µl
aliquots of the competent host cells and allowed to
stand on ice for 40 minutes. The contents of each tube
25 was then added to 200 µl of fresh E. coli JM109, 3 ml of
molten 0.7% Luria agar containing 10 µl of 100 mM
isopropyl-β-D-thio-galactopyranoside (IPTG) and 50 µl of
2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
(X-Gal). This mixture was plated on a Luria plate and
30 incubated overnight at 37°C. Four of the resulting
clear plaques were picked from the plates and grown in
10 ml cultures using JM109 as the host strain. Single
strand phage DNA was prepared from these cultures and
sequenced by the dideoxy method using M13 universal
35 primer. One of these four DNA's had the desired
sequence and was saved for assembly of the rb-bFGF gene
into the expression vector.

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Section II of the manufactured b-bFGF gene was
cloned into M13mp18 for sequencing using the same method
as for section I. In this case, the M13mp18 vector was
digested with EcoRI and HindIII (3-fold excess) in order
5   to accommodate the sticky ends of section II. For the
ligation, 0.025 pmole of M13mp18 vector was mixed with
0.075 pmole of the phosphorylated synthetic FGF section
II and incubated 4 hours at 14°C as before.
Transformation using the same $CaCl_2$ method resulted in
10   clear plaques as for Section I, but since a high
background of clear plaques was present on the control
plates, further selection by hybridization was carried
out. Several plaques were grown using JM109 host as
described before and supernatants containing phage DNA
15   were dotted on nitrocellulose filters. Oligonucleotides
18 and 24 used in the synthesis of Section II were
radiolabeled with ^{32}P-ATP using polynucleotide kinase
and were used to probe these filters. Two positive-
screening clones were selected and sequenced as before.
20   One of these clones had the expected sequence and was
used in the assembly of the gene into pCFM1156.

EXAMPLE 3

25   Assembly of rb-bFGF Gene in the Expression Vector
pCFM1156

Double-stranded replicative form DNA for the
section I and II M13 clones was prepared. 500 ml
30   cultures of each phage in JM109 host were grown and the
cells harvested by centrifugation. Cells were then
resuspended in 15% sucrose, 0.05 M tris, 0.05 M EDTA,
and 1 mg/ml lysozyme and incubated on ice for 25
minutes. RNAse was added to 0.1 mg/ml and incubation on
35   ice continued for 10 more minutes. An equal volume of
0.1% triton X-100, 50 mM tris, 50 mM EDTA was added and

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incubation on ice continued for another 10 minutes.
These lysates were then centrifuged for 60 minutes at
39000G and the clear supernatant saved. Ethidium
bromide was added to 1 mg/ml and cesium chloride was
5 added to give a density of 1.55 g/ml. This solution was
centrifuged for 18 hours at 45,000 rpm in a VTi-50 rotor
in order to reach equilibrium. The supercoil DNA band
from each tube was visualized by UV light and collected
with a syringe. The ethidium bromide was quickly
10 removed by extraction with butanol and the CsCl was
removed by extensive dialysis against 10 mM tris, 0.1 mM
EDTA. DNA stocks prepared in this way were used for
further cloning.

        Although numerous vectors may be employed to
15 express this DNA, an expression vector having a
regulated promoter and temperature inducible copy number
control gene is preferred in order to maximize product
yields. The expression plasmid pCFM1156 used in this
example may readily be constructed from a plasmid
20 pCFM836, the construction of which is described in
published European Patent Application No. 136,490. The
pCFM836 is first cut with NdeI and then blunt-ended with
T4 polymerase such that both existing NdeI sites are
destroyed. Next, the vector is digested with ClaI and
25 SacII to remove an existing polylinker before ligation
to a substitute polylinker as illustrated in Table V.
This substitute polylinker may be constructed according
to the procedure of Alton, et. al., supra. Control of
expression in the expression pCFM1156 plasmid is by
30 means of a foreshortened lambda P_L promoter, which
itself may be under the control of a C_{I857} repressor
gene (such as is provided in E. coli strain K12 ΔHtrp).

        The expression vector pCFM1156 was digested (2-fold
excess) with XbaI and HindIII in preparation for
35 ligation with FGF sections I and II. Section I and II
DNA stocks, prepared as described above, were digested

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(2-fold excess) with either XbaI and KpnI (section I) or
KpnI and HindIII (section II). All three digests were
loaded onto a 1.2% low melting agarose gel made in 50 mM
tris-acetate and electrophoresed for 3 hours at 60
5   volts. The gel was stained with 1 µg/ml ethidium
bromide solution and the bands were visualized under UV
light. The linearized vector band and the FGF section I
and II bands were excised from the gel with a scalpel,
placed in separate tubes and melted at 70°C for 15
10 minutes. Five microliters of the molten gel containing
linearized vector was added to 10 µl each of the molten
gel containing sections I and II and the mixture
equilibrated to 37°C. The molten gel was mixed quickly
with an equal volume of ice-cold 2X ligase buffer
15 containing 2 mM ATP, and 0.5 unit T4 ligase and
incubated overnight at 14°C. Aliquots of this ligation
mix were transformed into frozen competent E. coli FM6
host strain using a transformation protocol described by
Hanahan, J. Mol. Biol. 166, 557-580, (1983), grown 2.5
20 hours to allow expression of kanamycin resistance, and
plated on Luria plates containing 20 µg/ml kanamycin.
Plates were incubated at 28°C overnight. Colonies were
replica plated onto nitrocellulose filters and the
master plate was saved. Colonies on the filters were
25 grown to about 1 mm diameter at 28°C and then placed at
37°C overnight to increase plasmid copy number. The
filters were screened by hybridization with radiolabeled
oligonucleotide 18 (from gene synthesis) at 65°C in 6X
standar saline citrate buffer (SSC, 0.15 M NaCl, 0.015 M
30 Na citrate). Of the twenty-five positive clones
obtained, four were selected and grown in 500 ml
cultures. Replicative form DNA was prepared as

35

- 25 -

TABLE V

```
 1  ATCGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTACCAT
    TAGCTAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGCCATGGTA
```

```
 1 ClaI, 12 XbaI, 29 NdeI, 35 HincII, HpaI, 39 MluI, 47  EcoRI1,
  53 HgiCl KpnI, 57 NcoI StyI,
```

```
61  GGAAGCTTACTCGAGGATCCGCGGATAAATAAGTAACGATCC
    CCTTCGAATGAGCTCCTAGGCGCCTATTTATTCATTGCTAGG
```

```
 63 HindIII, 70 AvaI XhoI, 75 BamHI Xho2, 79 Sac2,
```

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described for sections I and II using a cleared lysate
procedure followed by CsCl equilibrium density gradient
centrifugation. These four clones were sequenced
directly using the expression vector's double stranded

5 form as a template for the dideoxy sequencing reactions,
and all four clones were found to have the correct
sequence. One of these clones was chosen to be utilized
in the expression of rb-bFGF and is hereinafter referred
to as pCFM1156/bFGF. The DNA sequence for the rb-bFGF

10 gene thus constructed in the pCFM1156/bFGF vector is
represented in Table I.

                        EXAMPLE 4

15 Expression and Purification of rb-bFGF

Expression

        An overnight culture of the production strain was
20 grown at 28°C in Luria broth (Luria broth: bactotryptone
   10 g/l, yeast extract 5 g/l, NaCl 5 g/l) containing
   20 µg/ml kanamycin, and was used to inoculate an 8-liter
   fermentation batch. The 8-liter batch media contained
   40 g yeast extract, 40 g glucose, 10 g sodium chloride,
25 and appropriate buffer salts, vitamin solution, and
   trace metals. A dual feed protocol was used. The
   initial feed (1 liter) contained 450 g glucose plus
   appropriate vitamins and salts. After growth to 20 OD a
   second feed was begun at a rate of 200 ml/hour and the
30 temperature was shifted to 42°C. This feed solution
   contained 200 g/l bacto-tryptone, 100 g/liter yeast
   extract, and 100 g/l glucose. Growth was continued for
   6 hours at 42°C with the cell concentration reaching 50
   OD at harvest.
35

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Purification

E. coli cells were broken in water by a Gaulin
homogenizer, and centrifuged at 4.2 K for 40 min with a
5 J6B centrifuge. When analyzed by SDS-PAGE, rb-bFGF
appeared in both pellet and supernatant, the protein in
the supernatant being 60-70%. The pellet was,
therefore, discarded and the supernatant was purified
using ion exchange chromatography. The supernatant,
10 after titration to pH 7.4 with Tris-base, was made 1 mM
DTT and mixed with a carboxymethyl cellulose-Sehparose®
resin (CM-Sepharose®, Pharmacia, Uppsala, Sweden)
equilibrated with 40 mM Tris-HCl/1 mM DTT/pH 7.4. The
resin was then washed batch-wise with the same buffer,
15 and eluted column-wise with a linear NaCl gradient from
0 to 0.7 M. A single peak around 0.5 M was pooled based
on SDS-PAGE analysis. The pool was titrated to pH 8.2
with Tris-base, diluted three-fold with cold water, and
loaded onto a CM-Sepharose in 40 mM Tris-HCl/1 mM DTT/pH
20 8.2. The column was washed with 0.15 M NaCl and eluted
with a linear NaCl gradient of 0.15 to 0.5 M. A major
peak between two small peaks was pooled and found to be
approximately 95% pure with a small amount of dimer when
analyzed by non-reducing SDS-PAGE.

25   The pool was immediately titrated to approximately
pH 5 with 1 M sodium acetate/pH 4 to prevent the rb-bFGF
product from oxidation and then directly loaded on a
Sephadex G-75 column in 20 mM Na citrate/0.1M NaCl/pH 5,
resulting in a single peak eluting between a shoulder
30 (corresponding to dimer) and a peak for small compounds
(such as DTT). The fractions in the peak were pooled
and stored at 4°C, -20°C or lyophilized. The yield in
this gel filtration was nearly 100%. Approximately
160 mg of rb-bFGF was obtained from 560 g of cell paste.
35

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TABLE VI

Human basic Fibroblast Growth Factor/peptide sequence

```
  1                          10                              20
MetProAlaLeuProGluAspGlyGlySerGlyAlaPheProProGlyHisPheLysAsp

                            30                              40
ProLysArgLeuTyrCysLysAsnGlyGlyPhePheLeuArgIleHisProAspGlyArg

                            50                              60
ValAspGlyValArgGluLysSerAspProHisIleLysLeuGlnLeuGlnAlaGluGlu

                            70                              80
ArgGlyValValSerIleLysGlyValCysAlaAsnArgTyrLeuAlaMetLysGluAsp

                            90                             100
GlyArgLeuLeuAlaSerLysCysValThrAspGluCysPhePhePheGluArgLeuGlu

                           110                             120
SerAsnAsnTyrAsnThrTyrArgSerArgLysTyrThrSerTrpTyrValAlaLeuLys

                           130                             140
ArgThrGlyGlnTyrLysLeuGlySerLysThrGlyProGlyGlnLysAlaIleLeuPhe

             146
LeuProMetSerAlaLysSer
```

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EXAMPLE 5

<u>Characterization of rb-bFGF</u>

5   The activity of rb-bFGF was examined in
^{3}H-thymidine incorporation on 3T3 cells. All the
preparations, stored at 4°C, and -20°C and lyophilized,
showed a dose-dependent activity with a protein
concentration from 20-210 pg/ml for half maximal
10   activity depending on the particular strain of 3T3 cells
utilized in the assay.

<u>General Characteristics of the Final Product</u>

15   280/260 ratio   ~2.0
       LAL endotoxin assay   <0.6 EU/ml   (0.623 FGF mg)
       DNA   <20 pg/ml   (0.623 FGF mg)
       Extinction coefficient   1.3 for 0.1% protein
       (278 nm)
20   Purity   >95%

<u>Stability</u>

        When a rb-bFGF preparation was incubated at 4°C at
25   different pH values, rb-bFGF showed formation of
polymers composed of more than one bFGF molecule at pH $\geq$
6.0 due to inter-chain disulfide bonds and degradation
at pH $\leq$ 4.0 due to acid instability of Asp-Pro bond.
Based on this information, the pH 5 buffer was
30   selected. This stability data suggests that free
sulfhydryl groups present in the final product tend to
form inter-chain, rather than intra-chain, disulfide
bonds. The rb-bFGF preparation is apparently monomeric
when determined by a Sephadex G-75 gel filtration.

35

- 30 -

Example 6

Structural Characterization of rb-bFGF

5   Purified rb-bFGF protein prepared as described in
Example 4 was examined for conformational analyses using
sulfhydryl titration, circular dichroism (CD) and gel
filtration. The cysteine residues in the molecule were
also studied by peptide mapping and sequence analysis.

10   **Methods**

       Ultra-pure urea was obtained from Schwarz/Mann
(Cleveland, Ohio). 5,5'-dithiobis-(2-nitrobenzoic acid)
15 (DTNB) was obtained from Sigma Chemical Company (St.
Louis, Missouri). Recombinant b-bFGF was purified from
E. coli cells as described in Example 4.
       Into 12 ml of rb-bFGF at 0.626 mg/ml in 20 mM Na
citrate, 0.1 M NaCl, pH 5.0 was added 1.5 ml of 0.5 M
20 iodoacetacetic acid in 1 M Tris-HCl, pH 8.2 (final
55 mM), then titrated to pH 8.2 with saturated Tris-
base. The mixture was allowed to stand for 12 hours at
room temperature, then dialyzed against cold 20 mM
sodium citrate, 0.1 M NaCl, pH 5.0. After exhaustive
25 dialysis, the protein solution was concentrated and
centrifuged to remove trace amounts of precipitates.
Titration of sulfhydryl groups was carried out using
DTNB essentially according to Habeeb, A.F.S.A. 25
Methods. Enzymol. 457-465 (1972).
30   Gel filtration was carried out on a Sephadex® G-75
column (2.5 x 110 cm, Pharmacia, Uppsala, Sweden) in 20
mM sodium citrate, 0.1 M NaCl, pH 5.0 at a flow rate of
1 ml/min. The column was calibrated with myoglobin
(17000) and bovine serum albumin (68000).
35   Protein concentration was determined
spectrophotometrically.

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UV absorbance spectra were determined on a Hewlett-Packard Model 8451A diode spectrophotometer. Circular dichroic spectra were determined at room temperature on a Jasco Model J-500C spectropolarimeter equipped with an Oki If 800 model 30 computer. Measurements were carried out at room temperature with cuvettes of 1 and 0.02 cm for near and far UV ranges, respectively. The data were expressed as the mean residue ellipticity, $[\theta]$, calculated using the mean residue weight of 112 for bFGF.

Trypsin digests were performed for 4 hours at 37°C in 0.1 M ammonium bicarbonate, pH 8.0, using tosylphenylalanyl chloromethyl ketone-treated trypsin (Cooper Biomedical, Malvern, Pennsylvania). The rb-bFGF concentration was 0.5 mg/ml, with the enzyme to substrate ratio being 1:50.

Peptide mapping was done by reverse phase HPLC chromatography using a Vydac C4 column (214TP54) (Vydac, Hesperia, California) run at a flow rate of 0.8 ml/min. The gradient ran from 0% buffer A (0.1% trifluoroacetic acid (TFA)/water) to 50% buffer B (0.1% TFA/90% acetonitrile/water) with a linear gradient of 90 minutes. Samples were injected under a Wisp® (Waters Corporation, Milford, Massachusetts) autosampler control. Fractions were collected by automatic peak detection monitoring at 220 nm.

Sequence analysis was performed on instruments from Applied Biosystems (Foster City, CA). Models 470A and 477A were used. In the 470A instrument, a modified R2 reagent was used (Lai, 163 Anal. Chim. Acta. 243-248 1984).

Results

Sulfhydryl titrations were carried out using the purified rb-bFGF preparation. When a sample, obtained

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by dialysis of rb-bFGF vs. 0.1 M Tris-HCl, pH 8.0,
containing 1.5 mM EDTA, was titrated with DTNB, the
mixture showed light scattering rendering the absorbance
measurement impossible. Therefore, the dialyzed rb-bFGF
5 sample was diluted with 4 volumes of 6 M guanidinium-HCl
(GdnHCl) in 0.1 M Tris-HCl, 1.5 mM EDTA, pH 8.0
immediately followed by DTNB titration. Titration of
this sample showed approximately 2.5 moles of free
sulfhydryl per molecule.

10   DTNB titration experiments were also carried out
with reduced rb-bFGF. Thus, rb-bFGF in 0.1 M Tris-HCl,
1.5 mM EDTA, pH 8.0 was first reduced with 50 mM DTT.
After 2 hours incubation, the reduced rb-bFGF was
precipitated with 5% trichloroacetic acid (TCA) and the
15 resultant pellet was washed 3 times with 5% TCA. The
final pellet was washed with a small amount of H_2O and
the protein solubilized with 6M GdnHCl in 0.1 M Tris-
HCl, 1.5 mM EDTA, pH 8.0 immediately followed by DTNB
titration. This resulted in detection of approximately
20 4 moles of free sulfhydryl per mole of reduced
rb-bFGF. This result, combined with about 2 detectable
free sulfhydryls in non-reduced sample, suggested that
the rb-bFGF preparation has two free sulfhydryl groups,
and that the other cysteines form a disulfide bond,
25 which can be readily cleaved without denaturants.
        Sulfhydryl titrations were also carried out with
the S-carboxymethylated (S-CM) rb-bFGF. The S-CM
rb-bFGF sample was dialyzed vs. 40 mM Tris-HCl, pH 7.5
and treated with 100 mM DTT overnight. The dialyzed S-
30 CM rb-bFGF was treated with 5% TCA and assayed for free
sulfhydryl groups as described above. The results
showed 2 moles of free sulfhydryls per mole of S-CM rb-
bFGF, indicating that the S-CM rb-bFGF has two free
sulfhydryl groups upon reduction. This suggested that
35 rb-bFGF had two free sulfhydryl groups that had been
carboxymethylated.

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Peptide mapping was employed to investigate whether
two cysteines are involved in a disulfide bond and two
are in the free reduced state. Digestion with trypsin
was used to isolate the cysteines into three peptides:
5   24-27, containing cys-26; 68-73, containing cys-70; and
88-98, containing cys-88 and cys-93.

Trypsin digestion was performed on unreduced native
rb-bFGF and on unreduced S-CM rb-bFGF. Following
digestion, the peptides produced were separated on
10   reverse phase HPLC, and the resulting peptide maps were
compared (Figure 5). The introduction of the acetate
group into a peptide containing cysteine was predicted
to make the peptide more hydrophilic, and hence elute
sooner in a reverse phase HPLC gradient.

15   In Figure 5, it is seen that two peptide peaks are
shifted to earlier elution times in the S-CM rb-bFGF
relative to the native rb-bFGF. One peak shifts from 25
minutes to 16 minutes and the other from 57 minutes to
53 minutes. These peaks were collected and identified
20   by sequence analysis. The sequence of the peptide
eluting at 25 minutes was determined and found to
correspond to the tryptic peptide 68-73. This peptide
contains cys-70. Since this peptide yielded a single
peak, it was presumed to be one of the cysteines not
25   involved in a disulfide bond.

The sequence of the peptide eluting at 53 minutes
gave two sequences. Since a single peak in this region
shifted on carboxymethylation and the peptide contained
within this region gave rise to two sequences, this was
30   assumed to be the peptide containing the disulfide
bond. Comparison of the two sequences obtained from the
53-minute peak with the whole FGF sequence indicated
that these two constituent sequences correspond to the
24-27 and 88-98 peptides. There are three cysteines in
35   these two peptides. Identification of
carboxymethylcysteine phenylthiohydantoin at the first

- 34 -

sequence cycle indicated cys-88 as the other free
cysteine and suggested that there is a disulfide bond
between cysteine 26 and 93.

5   The S-CM rb-bFGF was assayed in 3T3 cells as
described in Example 8. The results showed that the
S-CM rb-bFGF has an activity comparable to the native
molecule, demonstrating that the free sulfhydryl groups
in the molecule are not required for the mitogenic
activity on 3T3 cells.

10   Circular dichroic spectra were obtained with
rb-bFGF in 20 mM sodium citrate, 0.1 M NaCl, pH 5.0.
The results are shown in Figure 6. The near UV CD shows
intense negative ellipticities with a number of
extrema. Those extrema at 262 and 269 nm are due to
15   phenylalanine residues, and the extreme at 275 nm
probably arises from tyrosine absorbance (Strickland, 2d
CRC Crit. Rev. Biochem. 113-175 (1974)). A broad,
negative ellipticity can be seen from 306 nm down to 290
nm, which then overlaps with the aromatic CD. This CD
20   band is likely due to a disulfide, which usually has a
peak around 240 to 250 nm. Therefore, the observed near
UV CD of rb-bFGF may be characterized as a broad
negative disulfide CD, and several aromatic CD
signals. These results indicate that the aromatic
25   residues are in rigid, asymmetric environments and that
the rb-bFGF has a distinct tertiary structure
(Timasheff, Vol. II In the Enzymes, Boyer (ed.) 371-443
(1970).

The S-CM rb-bFGF was also examined by CD. The
30   results showed both near and far UV spectra identical to
those of the native protein, indicating that the
S-carboxymethylation of the two free sulfhydryl groups
does not apparently affect the secondary and tertiary
structures of the protein. This is consistent with the
35   observation that the S-carboxymethylation does not
affect the activity of the protein.

- 35 -

The far UV CD, shown in Figure 6, exhibits a
spectrum typical for unordered structure (Greenfield and
Fasman, Biochemistry 8, 4108-4116 (1969). The spectrum
exhibits a negative peak at 202 nm and a broad positive
5   peak around 225 nm. The 202 nm peak and almost no
negative ellipticities between 205 and 220 nm are
characteristics typical for unordered secondary
structure. The positive peak at 225 nm is likely due to
contribution from β-turns (Chang et al., 91 Anal.
10 Biochem. 13-31 1978). Therefore, the polypeptide of
rb-bFGF seems disordered, but folded into a distinct
tertiary structure with β-turns.

Gel filtration of rb-bFGF in 20 mM sodium citrate,
0.1 M NaCl, pH 5.0 showed that the protein elutes at the
15 same elution volume as that for myoglobin (17000).
Since the molecular weight of bFGF (16000) is smaller
than that of myoglobin, the gel filtration result
suggests a slightly asymmetric conformation of the
protein.
20

EXAMPLE 7

HUV-EC Bioassay for rb-bFGF

25   Human umbilical vein endothelial (HUVE) cells used
in this experiment were isolated by Judith A. Berliner
by the method of Gimbrone et al, Human Vascular
Endothelial Cells in Culture, J. Cell Biol., 60, 673-684
(1974). Cells were maintained by subculturing into
30 culture flasks coated with 0.1% gelatin in phosphate
buffered saline, and 10 μg/ml fibronectin (Boehringer
Mannheim, Ingelheim, West Germany) in media minus fetal
bovine serum for 30 minutes each, consecutively. Cells
were released with 0.0125% trypsin-0.005% EDTA and
35 passed 1:2 or 1:3 once a week. The maintenance media
used was MCDB105 (Irvine Scientific, Irvine, California)

with penicillin G (10 units/ml) streptomycin (10 ug/ml),
fetal bovine serum (20%, hyclone), L-glutamine (2mM),
sodium pyruvate (1mM), heparin (40 µg/ml, 170 units/mg)
and endothelial cell growth supplement (ECGS, 40 ug/ml

5 Collaborative Research). Cells were grown in a 2% CO_2
incubator.

The following experiment was carried out to compare
the growth sustaining and mitogenic activities of three
different forms of FGF on HUV endothelial cells:

10 1) E. coli derived recombinant bovine basic FGF
(rb-bFGF); 2) bovine acidic FGF (b-aFGF, particularly
purified from bovine brain); and, 3) natural bovine
basic FGF (bFGF purified from bovine pituitary glands,
Sigma Chemical Company, St. Louis, Missouri).

15 One hundred cells per well were plated into the
center 8 wells of four 24-well plates. One of the three
fibroblast growth factors identified above was added to
each well, except in the case of the control wells, to
which no growth factor was added.

20 The cells were fed three and six days after the
initial seeding with their same growth factor. Ten days
after the seeding, cultures were analyzed by crystal
violet straining.

The results in Table VII illustrate that there were
25 20% and 32% more colonies in the 8 wells which contained
rb-bFGF than colonies in the wells containing either
aFGF or natural bFGF, respectively. Also of interest is
the fact that 75% of the total colonies arising from
culture with rb-bFGF were 0.5 mm and larger, while only
30 55% and 51% of the colonies grown with aFGF and n-bFGF,
respectively were that size.

35

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Table VII

Growth Factor	# of Colonies /8 Wells	# of Colonies ≥ 0.5 mm/8 Wells
rb-bFGF	351	265
aFGF	293	160
natural bFGF	265	134
Control	8	0

EXAMPLE 8

rb-bFGF Bioassay on NIH 3T3 Cells

Cells used for this assay were NIH3T3 cells from ATCC. The cells were grown in DME medium with penicillin G (10 µg/ml), streptomycin (10 µg/ml), and calf serum (10%). The cells were passed 1:40, two times a week. On day 1 of the assay, subconfluent cultures were trypsin dispersed and plated into 24-well plates at a concentration of 2×10^4 cells per ml, 1 ml per well in the above growth media.

On day 5, the media was replaced with DME containing penicillin streptomycin and 5% human platelet poor plasma (cleared heparinized serum), 1 ml per well. On day 6, experimental and control samples of FGF were added to the media in volumes no greater than 100 µl.

Eighteen hours later, the cells were pulsed for 1 hour with 1 ml of DME containing 5% calf serum and 2 µCi of ^{3}H-Me thymidine at 37°C. The cells were then washed one time each with 1 ml of phosphate buffered saline (PBS) and 5% trichloroacetic acid, both at 4°C. Plates were allowed to air dry for 30 minutes, after which 1 ml of 0.25 M NaOH was added to each well. After one hour

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at room temperature, the contents of each well were
transferred to a separate counting vial containing 10 ml
of Aquasol® II (New England Nuclear, Boston,
Massachusetts). Samples were counted for 1 minute
5   through the 0-397 window of a Beckman LS 7,500
scintillation counter (Beckman Instruments, Inc.,
Fullerton, California).

Recombinant b-bFGF standards were made from a stock
with a concentration of 600 μg/ml in a sodium citrate
10   buffer of pH 5. The range of standards used was 5 pg to
1,000 pg per ml. The standard of lowest concentration
which gave maximal ^{3}H-thymidine uptake was 500 pg. An
average of 140-210 pg gave the half maximal
incorporation of labelled thymidine.
15

μ   Example 9

Construction of the rh-bFGF gene

20   Conversion of the bovine bFGF gene prepared in
Example 1 to a gene encoding for h-bFGF was accomplished
by oligo site directed mutagenesis.

The segment to be modified was first cloned into
the phage vector M13mp18 and transformed into E. coli
25   JM101 for growth and preparation of single stranded
phage DNA (Messing, J. Vol. 9, Nucl. Acid Res., 309-321
(1981)). Approximately 0.5 μg of template DNA was mixed
with 5 pmol of universal M13 sequencing primer and 5
pmol of each mutagenic primer, heated to 65°C for 3
30   minutes and allowed to slow cool. The annealed
template-primer was then mixed with ATP, a
deoxynucleotide triphospate (dNTP) mix, DNA polymerase I
(DNA PolI) large fragment (Klenow fragment), and ligase
followed by incubation at 15°C for 4 hours. Aliquots of
35   this reaction mix were transformed into competent
E. coli JM101 cells and plated in 0.7% Luria agar.

Plaques containing mutant phage were selected by
hybridization of replica nitrocellulose filters with
^{32}P-labeled mutagenic primer. Single strand DNA was
prepared from positive-screening plaques and its

5 sequence verified using the dideoxy chain-termination
method. The amino acid changes made and the
corresponding mutagenic primers used were:

     pro-129 to ser-129) 5' GACCAGTCTTCGAACCCAGTTTGTA 3'
     ser-113 to thr-113) 5' CATACCAGGAAGTGTATTTACGAGA 3'

10   The primers correspond to the antisense strand of
the bFGF gene.

Example 10

15 <u>Purification of rh-bFGF</u>

    <u>E. coli</u> cells containing the synthetic h-bFGF genes
in pCFM1156 were grown as described above in
Example 4. After disruption of the cells and low speed

20 centrifugation, the resultant rh-bFGF (Table VI) was
found both in the supernatant and pellet fractions.
Purification from the pellet requires solubilization by
denaturants followed by refolding to obtain active
material. These steps can be avoided by purification

25 from the supernatant fraction as described below. This
fraction was applied to a CM-sepharose column in 40 mM
Tris-HCl, pH 7.4 and eluted with a linear NaCl
gradient. The fractions containing rh-bFGF were then
bound to the same resin, but in 40 mM Tris-HCl, pH 8.2

30 and again eluted with a linear NaCl gradient. In these
two chromatographies, 1 mM DTT was included to prevent
oxidation, which otherwise resulted in the formation of
intermolecular disulfide bonds. The protein was further
purified by gel filtration using a Sephadex® G-75 column

35 (Pharmacia, Upppsala, Sweden) in 20 mM sodium citrate,
0.1 M NaCl, pH 5.0. Initial attempts to purify rb-bFGF

- 40 -

from E. coli cells showed that dimer was readily formed
when 1 mM DTT was not included throughout the
purification process. Purification of the human bFGF
was carried out in essentially the same manner as for
5   the bovine material as set forth in Example 4. No
difference between rh-bFGF and rb-bFGF was noted in any
of the purification steps. When examined on SDS-PAGE
under reducing conditions, the r-bFGFs gave a major band
at 16,500 daltons corresponding to the monomeric
10   molecular weight and minor bands at higher molecular
weights which probably represent dimer and tetramer
forms. (See Fig. 4.) When run under non-reducing
conditions, more contamination by the higher molecular
weight bands was apparent. Amino acid sequence analysis
15   of the purified rh-bFGF revealed that methionine had
been cleaved from most of the material, yielding 70%
proline, 13% alanine, and only 17% methionine on the N-
terminus. As does the natural bFGF, rh-bFGF exhibits a
strong affinity for heparin, eluting from heparin
20   sepharose columns at approximately 1.5-2.0 M NaCl (data
not shown).

Example 11

25   Characterizations of rh-bFGF

The activity of rh-bFGF was examined in ^{3}H-
thymidine incorporation on 3T3 cells as described in
Example 8. As shown in Fig. 3, rh-bFGF shows
30   essentially identical potency, in this assay, as
rb-bFGF, producing half-maximal stimulation of DNA
synthesis at a dose of about 150-200 pg/ml.

35

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Example 12

HUV-EC BioAssay for rh-bFGF

5   The HUVE cell assay is described in Example 7, with
minor variations in the plating and time of assay.
Addition of rh-bFGF resulted in extensive cell
proliferation in comparison with controls containing no
growth factor. No significant difference between
10   recombinant bovine and human bFGF was observed.

Table VIII

Growth Factor	Total Colonies	Large Colonies
rh-bFGF	76	22
rb-bFGF	68	19
Control	23	0

20   All fibroblast growth factors were added at a
concentration of 10 ng/ml at the time of plating and on
days 3 and 6 after plating. Cells were stained and
counted on day 9. Colonies greater than 0.5 mm in
diameter were scored as large colonies.
25

Example 13

Preparation of rh-bFGF Analogs

30   In order to improve stability and facilitate
purification of rh-bFGF, oligonucleotide site-directed
mutagenesis was used to modify the human bFGF gene so
that the sequences which coded for some or all of the
cysteine residues would code for serine instead. It is
35   recognized that the bovine bFGF gene can be modified in
the same manner and that the sequences which coded for

one or all of the cysteine residues could code for other
amino acids, such as, alanine, aspartic acid and
asparagine. Four oligonucletoides were synthesized,
each designed as shown in the table below:

5

Table IX

10

Oligonucleotide	Sequence	coding change
102-21	5' ACCGTTTTTGGAGTACAGACG 3'	CYS TO SER AT POSITION 26
102-22	5' CCGGTTAGCAGATACACCTTT 3'	CYS TO SER AT POSITION 70
102-23	5' GTCAGTAACAGACTTAGAAGC 3'	CYS TO SER AT POSITION 88
102-24	5' GAAAAAGAAAGATTCGTCAGT 3'	CYS TO SER AT POSITION 93

15

20

25

The first mutagenesis used oligonucleotides 102-22
30 and 102-23 to convert the cysteines at positions 70 and
88 to serine. Subsequently, genes were constructed in
which all 6 possible pairs of cysteines are replaced by
serines and one in which all 4 cysteines are replaced by
serines. Oligonucletoides used in the construction of
35 these mutant genes are listed below:

- 43 -

Table X

Analog	oligonucleotides used for mutagenesis
serine 70,88	102-22, 102-23
serine 26,93	102-21, 102-24
serine 26,70	102-21, 102-22
serine 26,88	102-21, 102-23
serine 70,93	102-22, 102-24
serine 88,93	102-23, 102-24
serine 26,70,88,93	102-21, 102-22, 102-23, 102-24

All mutagenesis reactions were carried out in
essentially the same manner, differing only in the
oligonucleotides used for the reaction and for screening
of the resultant plaques by hybridization. Ten pmole
each of the M13 universal primer and the above primers
were phosphorylated by incubation with 1 mM ATP and 10
units of polynucleotide kinase in 10 µl of 70 mM tris,
10 mM MgC12, 5 mM DTT for 30 minutes at 37°C. Five
pmole of each kinased oligonucleotide was mixed with
about 0.5 µg of single stranded M13mp18/rb-bFGF, heated
to 60°C, and allowed to renature by cooling to room
temperature. To this template/primer mix was then added

1 μl of a solution 25 mM each in dATP, dCTP, dGTP, and
TTP, 1 μl of 100 mM ATP, 2 units T4 ligase, and 8 units
DNA PolI large fragment. This mixture was incubated at
14°C for 4 hours. Aliquots of the ligation mix were

5 transformed into E. coli JM101 made competent by
treatment with 50 mM CaCl2 and plated in 0.7% Luria agar
on previously poured 1.5% Luria agar plates. Lifts onto
nitrocellulose filters were performed on the resulting
clear plaques and filters were screened by hybridization

10 to the appropriate radiolabeled oligonucleotide.
Several positives were obtained using each probe.
Positive plaques were picked, and single strand DNA was
prepared for use as a template in the second round of
mutagenesis. When the desired construction was

15 obtained, replicative form DNA was purified by cell
lysis and banding in a CsCl density gradient. The
modified genes were then transferred to the plasmid
vector pCFM1156 for expression in E. coli. The modified
gene was excised from its M13 vector by cleavage with

20 XbaI and HindIII and purified by agarose gel
electrophoresis. This purified fragment was then
ligated into XbaI/HindIII cut pCFM1156. The new
constructions were transformed into E. coli strain FM5
for expression. The serine-70,88 and serine-26,93

25 analogs were transferred to the expression vector.
Growth of the production strain and subsequent
purification of the serine-70,88 analog was carried out
exactly as for the rb-bFGF and rh-bFGF as described in
Example 4, except that DTT was omitted from all

30 purification steps. The omission of DTT was possible
since the cysteine residues responsible for dimerization
of the bFGF during purification had been removed. Thus,
the serine-70,88 analog represents a significant
improvement over the r-bFGF with the natural sequence

35 since it contains no detectable dimer impurity and will
not tend to form dimer over time because it lacks free

- 45 -

sulfhydryl groups. Further, since the need for added
reducing agent is eliminated, possible formulation
problems may be avoided.

The serine-26,93 analog did not behave in the same
5 manner as the recombinant bovine and human bFGF and
serine-70,88 molecules, probably because it does not
have the disulfide structure of natural bFGF. During
purification a significant portion of the serine-26,93
analog was degraded. Such degradation also occured when
10 attempts were made to purify the rb-bFGF in the presence
of 7M urea, which denatures the molecule's tertiary
structure. Although the serine-26,93 analog does not
appear to have the same biological activity as the
serine-70,88 analogs when purified from the supernatant
15 as specified in Examples 4 and 11, the inactive analogs
may have application as antagonists or blocking
molecules.

Example 14

20

Purification of a serine-26,70,88,93 analog from
inclusion bodies

It was surprisingly found that when a serine-
25 26,70,88,93 analog was purified from inclusion bodies,
rather than from the supernatant, an active analog was
obtained, despite alteration of apparent non-free
sulfhydryls.

Using oligo site-directed mutagenesis, the
30 synthetic gene for h-bFGF was modified generally as
described in Example 9 to replace all four cysteine
codons with nucleotides coding for serine residues. The
corresponding protein was expressed in E. coli and
purifed from inclusion bodies by solubilization in urea
35 followed by a series of column chromatographies and a
folding step. The resulting protein, having no cysteine

- 46 -

residues, is unable to form either intramolecular or intermolecular disulfide bonds. Nevertheless, this analog protein was found to exhibit mitogenic activity on NIH 3T3 cells indistinguishable from that exhibited
5   by the natural sequence.

Oligo site-directed mutagenesis

A previously constructed bFGF analog gene in which
10 codons for cysteines 70 and 88 had been converted to serine codons was used as the starting template for the mutagenesis. Approximately 0.5 µg of template DNA was mixed with 5 pmol universal M13 sequencing primer and 5 pmol of each mutagenic primer, heated to 65°C for 3
15 minutes and allowed to slow cool. The annealed template-primer was then mixed with ATP, a dNTP mix, DNA PolI large fragment, and ligase, followed by incubation at 15°C overnight. Aliquots of this reaction mixture were transformed into competent JM101 cells and plated
20 in 0.7% Luria agar. Plaques containing mutant phage were selected by hybridization of replica nitrocellulose filters with each ^{32}P-labeled mutagenic primer. Single strand DNA was prepared from plaques which scored positive in both hybridization screens. Selection of
25 the desired sequence was verified using the dideoxy chain-termination DNA sequencing method. The amino acid changes made and the corresponding mutagenic primers used were:

        cys-26 to ser-26   5' ACCGTTTTTGGAGTACAGACG 3'
30   cys-93 to ser-93   5' GAAAAAGAAAGATTCGTCAGT 3'
        Both primers correspond to the antisense strand of the bFGF gene.

35

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Expression and Purification.

    E. coli cells harboring the plasmid containing the
mutant bFGF gene were grown in 2x Luria broth at 30°C to
5 approximately 0.5 A600, than shifted to 42°C to induce
expression of bFGF. After growth overnight, the cells
were harvested by centrifugation and lysed by three
passages through a French Press at 10,000 psi. The
insoluble material containing the bFGF trapped in
10 inclusion bodies was collected by low speed
centrifugation. The inclusion bodies were solubilized
in urea and subjected to cation exchange and silica
column chromatographies. The partially purified analog
was folded by dilution and then purified to near
15 homogeneity by cation-exchange column chromatography.
Recombinant natural sequence human bFGF was purified
from the soluble fraction of lysed E. coli cells by a
series of chromatographic steps.

20 Mitogenesis Assay on NIH 3T3 Cells

    The biological activity of the analog bFGF was
tested by its ability to stimulate ^{3}H-thymidine uptake
in confluent cultures of NIH 3T3 cells, as described in
25 Example 7. Figure 7 shows the concentration dependence
of mitogenic acitivity for the serine-26,70,88,93 analog
and for natural sequence h-bFGF. Within experimental
error, the two profiles are indistinguishable, with the
half-maximal mitogenic effect observed at a dose of
30 about 150 pg/ml. These results indicate that the
conformation required for receptor-mediated mitogenic
acitivity is not altered by the substitution of the four
cysteine resides, at least where the analog is purified
from inclusion bodies, rather than from the supernatant.
35

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Example 15

Bioactivity of rh-bFGF analogs

5   The biological activity of the rh-bFGF analogs was
characterized as described for the bovine form in
Examples 7 and 8. The half-maximal activity of the
rh-bFGF serine-70,88 analog in the mitogenic assay on
NIH3T3 cells (example 8) is identical to that of the
10 rb-bFGF and rh-bFGF as shown in Figure 3. The serine-
26,93 analog showed no activity in this assay, as
expected.

      The serine-70,88 analog was also tested for its
ability to support the growth of HUVE cells as described
15 in Example 7. The rh-bFGF serine-70,88 analog was
equally as effective as the rb-bFGF and rh-bFGF in
promoting the growth of HUVE cells in culture.

Table XI

20

Growth Factor	Total Colonies	Large Colonies
Ser-70,88 rh-bFGF	84	24
rh-bFGF	76	22
rb-bFGF	68	19
Control	23	0

Example 16

30

In vivo study of efficacy of serine-70,88 analog

Pre-operative Preparation

35   New Zealand White rabbits, weighing approximately 3
kg each were anesthetized using 5 mg/kg Rompun®

(Farbenfabriken, Bayer, West Germany) as a sedative, followed (10 minutes later) by approximately 50-60 mg/kg ketamine, both administered intramuscularly. Each rabbit's weight was measured and recorded. A small

5   cotton or gauze plug was inserted into both ears of each rabbit, after which the inner surface and outer edges of both ears were shaved using an animal clipper (#40 blade). Commercially available Neet® depilatory cream was then applied to the inner surface of each ear for 10

10 minutes, after which time it was removed with dry gauze. The inner surface of the ears was wiped with saline-soaked gauze followed by application of a 70% alcohol solution. The dermis of the inner surface of one ear of each rabbit was blanched by infiltration of

15 the ear with a 2% xylocaine solution containing 1:1000 epinephrine (this requires 1.5-3 cc total volume) using a 30 gauge needle. The infiltrated area was then scrubbed with 3 cycles of betadine followed by the 70% alcohol solution. Where necessary, the ear plugs were

20 replaced with dry plugs at this point.

The rabbits were then transferred to a sterile surgical room. The blanched ear was immobilized on a plexiglass "ear board" (Washington University Medical Center, Division of Technical Services) which utilizes

25 two bar clamps, one at the tip and one at the base of the animal's ear, to stabilize the rabbit ear without compromising its blood supply. The animal was draped, and the surgical field (i.e., the inner surface of the blanched ear) sprayed with Betadine and allowed to dry

30 for 3-5 minutes.

Wounding

Sterile technique was employed throughout the

35 wounding procedure. The surface of the inner ear was scored gently with a 6 mm biopsy punch, and the biopsy

- 50 -

site cleared of all tissue and fibers (including the
periosteal membrane) down to the level of bare
cartilage, using micro-surgical forceps, tenotomy
scissors, a blunt edged 2 mm Lempert periosteal
5   elevator, and sterile cotton-tipped applicators.
Biopsies in which the cartilage was completely cut
through by the punch were not used for experimental
purposes. However, partial thickness scores of the
cartilage were considered acceptable. The location of
10 any nicks or natural holes in the cartilage was
carefully noted and recorded (for reference on the
harvest day). Blood was removed from the biopsy site
with sterile, cotton-tipped applicators, with care taken
to avoid excess blood in the wound. Each completed
15 biopsy was covered with a small piece of saline-soaked
gauze. Four viable biopsies were placed on each wounded
ear, two on each side of the midline (as defined by the
fold in the ear when it was stabilized upon the
board). In any event, no more than 5 total biopsies
20 were placed on each ear. The biopsies were positioned a
minimum of 1 cm apart.

Upon completion of one ear, the ear was covered
with saline-moistened gauze and then taped shut around
the gauze to retain moisture until application of FGF.
25 The second ear was then blanched, scrubbed, immobilized,
and wounded in the same manner as the first ear. Blood
was removed from the biopsy site of each second ear and
each completed biopsy covered with a small piece of
saline-soaked gauze. Upon completion of the second ear,
30 it also was covered with saline-moistened gauze until
application of FGF. Any rabbit that showed evidence of
recovery from anesthesia at any time prior to this point
in the procedure was reanesthetized with 25 mg/kg
ketamine, administered intramuscularly.
35

- 51 -

Application of FGF Preparations

FGF was applied first to the first wounded ear,
with the moist gauze being removed from the first ear
5   only and the unwounded surfaces of the ear gently wiped
dry with gauze. All biopsies were gently cleared of any
blood or excess fluid with sterile, cotton-tipped
applicators. The unwounded surfaces of the ear were
painted with tincture benzoin compound, carefully
10 avoiding the biopsies, and allowed to air dry for 3-5
minutes.

5 µg of either rb-bFGF or ser-70,88 rh-bFGF in
Zyderm® (Collagen Corporation) was applied to each
biopsy using a 26 gauge needle permanently mounted on a
15 low dead space 0.5 cc or 1 cc syringe (Becton-
Dickinson), or a micropipetter. The biopsy was able to
accomodate a maximum of 0.025 cc of the viscous bFGF-
Zyderm® preparation. After applying FGF to one biopsy,
it was immediately covered with the occlusive dressing
20 Tegaderm® (3M Corporation, Minneapolis, Minnesota),
being careful not to form any air bubbles or wrinkles
over the biopsy site. Tegaderm® was precut to an
approximate size of 2 cm^2. This process was repeated
for each biopsy on the ear. Any failed biopsies were
25 also covered with Tegaderm® to minimize risk of
infection. Following completion of the first ear, the
moist gauze was removed from the remaining ear, and the
procedure repeated, making sure to gently clear all
biopsies of any blood or excess fluid with sterile,
30 cotton-tipped applicators.

The rabbits were allowed to recover from anesthesia
under the observation of the investigator performing the
surgery. Upon recovery, a plastic collar extending
approximately 15-25 cm outward was placed around each
35 rabbit's neck, to prevent the rabbit from disrupting the
wounds or dressings. The rabbits were returned to an

- 52 -

isolation cage where they were maintained until
harvest. The wounds of any rabbits which had removed
their collars, and any wound on which the Tegaderm® had
been disrupted in some way prior to the harvest date,
5 were re-evaluated as soon as the problem was noted, and
discarded from analysis if the wounds appeared to be
damaged.

Harvest

.10

On the seventh day post-wounding, the rabbits were
anesthetized in the same manner as described for pre-
operative preparation. Each rabbit's weight was
measured and recorded, and a qualitative description of
15 the condition of the wounds was recorded, noting in
particular the presence or absence of the Tegaderm® and
of any excess fluid under the dressing. The rabbits
were sacrificed with 50 cc/kg air embolism administered
by intracardiac injection; both ears were then amputated
20 from the body using a #15 surgical blade mounted on a
knife handle.
Each biopsy, with approximately 5 mm of surrounding
tissue on any side and the Tegaderm® still intact, was
excised from the ear, and the biopsy site measured in
25 order to bisect it accurately at the midline, making
reference to notes taken on the day of wounding to avoid
bisecting through natural holes or nicks in the
cartilage. The biopsy was carefully bisected with a
single edge razor blade, using a single downward motion
30 to avoid disrupting the wound orientation. The bisected
biopsies were immediately placed in cassettes labeled
with the rabbit identification number, and placed in the
appropriate fixative for subsequent histologic
processing and quantitative analysis.
35

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Quantitative Histological Analysis

Carefully oriented cross sections through bisected
wounds were embedded, sectioned, and stained, using
5   either a mixture of Hematoxylin and Eosin or
Trichrome.   Rough cutting of the section was minimized
in order to obtain a cross section thrugh the true wound
center.   Measurements made include the
reepithelialization gap (EG) across the wound, the
10   maximum height (MH) of granulation tissue at the wound
at the wound edges (the average of both sides), and the
granulation tissue gap (GTG) across the wound, as shown
in Fig. 8.   Measurements were made blindly on precoded
slides by two independent observers using a calibrated
15   lens micrometer and converting to millimeters (mm).

The average of both observers' measurements were
calculated, after which the code was broken and the data
statistically analyzed.   If there was greater than 10%
difference between observers, the slide was
20   reanalyzed.   The resulting data is shown in Table XII.

25

30

35

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Table XII

rb-bFGF

	Control	rb-bFGF
5 Number of wounds	41	22
Frequency of total reepithelialization	24%	50%
GTG	4.68±0.12	4.66±0.13
10 MH of new granulation tissue (mm)	0.76±0.03	0.79±0.02

ser-70, 88 rh-bFGF analog

	Control	ser(70,88) rh-bFGF
15 Number of wounds	126	13
Frequency of total reepithelialization	22%	69%
GTG	4.41±0.09	3.85±0.14
20 MH of new granulation tissue (mm)	0.60±0.01	0.74±0.04

The data is presented as the mean ± standard error.

Both the rb-bFGF and ser-70,88 rh-bFGF in Zyderm®
preparations had a significant positive effect on
25 reepithelialization, although the effect with ser-70,88
rh-bFGF was somewhat greater. However, the formulation
of new granulation tissue was significantly affected by
ser-70,88 rh-bFGF, while rb-bFGF appeared to have no
effect. A possible explanation for this difference is
30 the enhanced stability of the ser-70,88 rh-bFGF analogue
compared to the natural sequence material. Since the
FGF is applied only once in this study, i.e., at the
time of wounding, stability in Zyderm® collagen could be
critical to the production of the positive effect on
35 granulation tissue formation. Other wound healing and
surgical applications using the bFGf analogs of the

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present invention will be apparent to those skilled in
the art.

    Numerous modifications and variations in the
practice of the invention will also be apparent to those
5 skilled in the art upon consideration of the foregoing
illustrative examples. Consequently, the invention
should be considered as limited only to the extent
reflected by the appended claims.

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WHAT IS CLAIMED IS:

1.   An analog of basic fibroblast growth factor
which differs from naturally occurring basic fibroblast
5   growth factor in terms of the identity and/or location
of one of more amino acid residues, wherein at least one
of the cysteine residues of said naturally occurring
basic fibroblast growth factor is is replaced by a
residue of a different amino acid.
10

2.   The analog of Claim 1 wherein said different
amino acid is selected from the group consisting of
serine, alanine, aspartic acid and asparagine.

15   3.   The analog of Claim 2 wherein, said different
amino acid is serine.

4.   The analog of Claim 1 wherein at least one of
said replaced cysteine residues is a cysteine residue
20   existing as a free sulfhydryl.

5.   The analog of Claim 1 wherein two of said
cysteine residues are replaced by a residue of a
different amino acid.
25

6.   The analog of Claim 5 wherein two of said
replaced cysteine residues are cysteine residues
existing as free sulfhydryls.

30   7.   An analog of basic fibroblast growth factor
having the amino acid sequence set forth in Table VI and
allelic variants thereof, wherein at least one of the
cysteine residues at positions 26, 70, 88, and 93, is
replaced by a residue of a different amino acid.
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8. The analog of Claim 7 wherein at least one terminal amino acid residue is deleted while said analog substantially retains the biological activity of naturally occurring basic fibroblast growth factor.

5

9. The analog of Claim 7 wherein said different amino acid is selected from the groups consisting of serine, alanine, aspartic acid, and asparagine.

10   10. The analog of Claim 8 wherein said different amino acid is serine.

11. The analog of Claim 7 wherein at least one of said replaced cysteine residues is selected from the

15   group consisting of cysteines at amino acid positions 70 and 88.

12. The analog of Claim 7 wherein two of said cysteine residues is replaced by a residue of a

20   different amino acid.

13. The analog of Claim 11 wherein said replaced amino acids comprise cysteines at amino acid positions 70 and 88.

25

14. The analog of Claim 11 wherein said replaced cysteine residues comprise cysteines at amino acid positions 26 and 93.

30   15. The analog of Claim 12 wherein said replaced cysteine residues comprise cysteines at amino acid positions 26, 70, 88, and 93.

16. A DNA sequence encoding for procaryotic or

35   eucaryotic expression of an analog of a basic fibroblast growth factor of Claim 7.

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17. The DNA sequence of Claim 16 wherein said DNA sequence is modified from human fibroblast growth factor gene.

5   18. The DNA sequence of Claim 17 wherein said human basic fibroblast growth factor gene is modified by oligonucleotide site-directed mutagenesis.

19. The DNA sequence of Claim 18 wherein said
10 human basic fibroblast growth factor is modified from a bovine basic fibroblast growth factor gene.

20. The DNA sequence of Claim 19 wherein said bovine fibroblast growth factor gene is modified by
15 oligonucleotide site-directed mutagenesis.

21. A pharmaceutical composition comprising a therapeutically effective amount of a basic fibroblast growth factor analog according to Claim 1 and
20 pharmaceutically acceptable adjuvants.

22. A method for treating a wound comprising administering to said wound a therapeutically effective amount of a basic fibroblast growth factor analog
25 according to Claim 1.

23. The method of Claim 22 wherein said wound is a surface wound.

30   24. The method of Claim 22 wherein said wound is a surgical wound.

25. The method of Claim 22 wherein said wound includes a bone fracture or defect.
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26. The method of Claim 22 wherein said wound includes a damaged nerve.

27. A method for generating tissue and/or organs
5   comprising administering a therapeutically effective amount of a basic fibroblast growth factor analog according to Claim 1.

28. A procaryotic or eucaryotic host cell
10   transformed or transfected with DNA according to Claim 16 in a manner allowing the host cell to express a basic fibroblast growth factor analog of Claim 7.

29. A method of producing a purified and isolated
15   basic fibroblast growth factor analog comprising the steps of:
       transfecting or transforming host cells with DNA according to Claim 16;
       culturing the transfected or transformed host cells
20   to allow the host cells to express a basic fibroblast growth factor analog; and,
       isolating said basic fibroblast growth factor analog.

25   30. A method for the purification of a recombinant basic fibroblast growth factor analog according to Claim 7 comprising subjecting a supernatant containing basic fibroblast growth factor analog to non heparin chromatography.
30

31. A method for the purification of a recombinant basic fibroblast growth factor analog according to Claim 7 comprising the steps of:
       solubilizing the inclusion bodies from host cell
35   cultures containing basic fibroblast growth factor; and,
       subjecting the solubilized inclusion bodies to non heparin chromatography.

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FIG. I
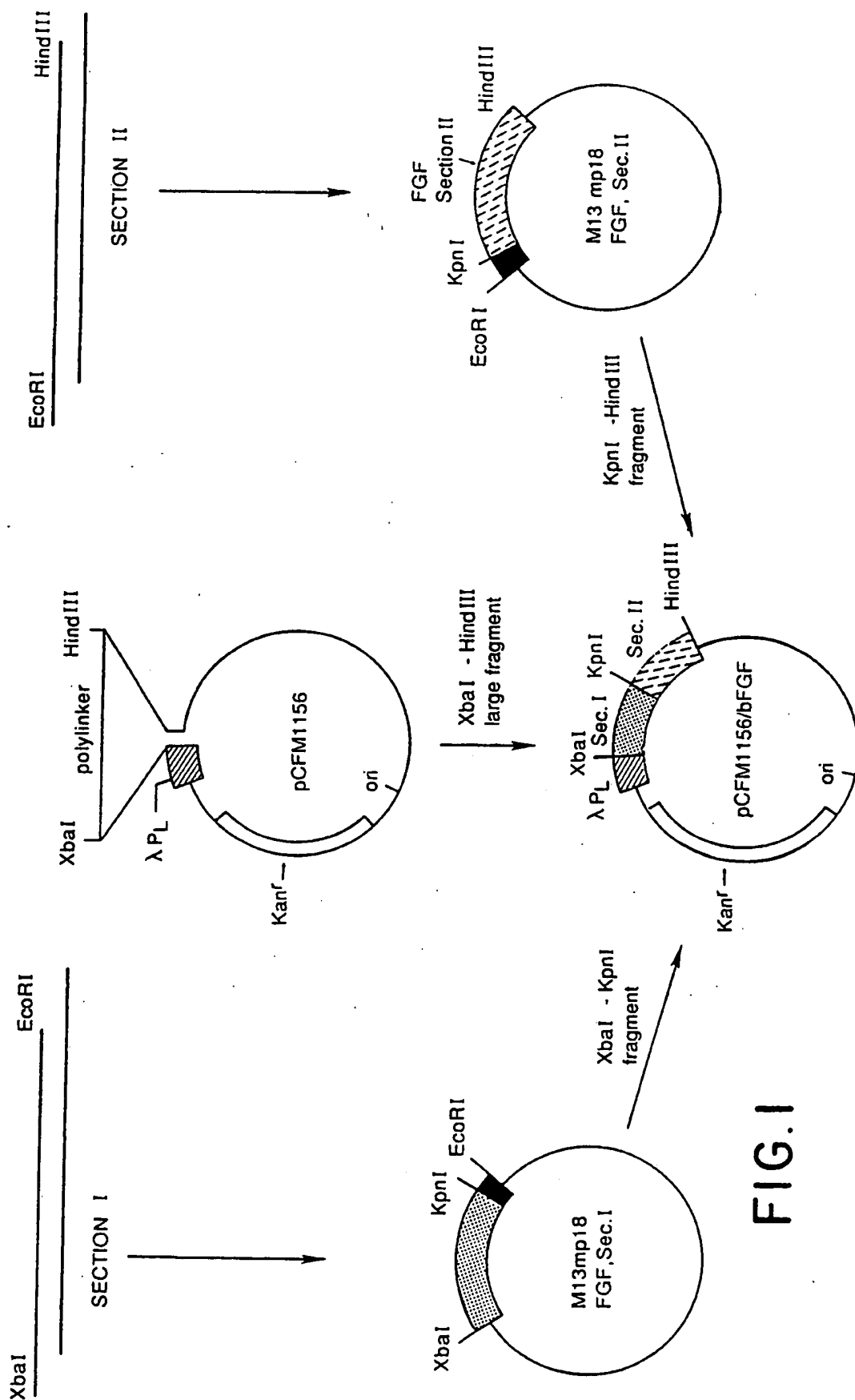
2/8

```
                                1
                                Met Pro Ala Leu Pro Glu Asp Gly Gly
          TCTAGAAGGAGGAATAACAT  ATG CCA GCT CTG CCA GAA GAT GGT GGA
              Xba I
```

```
      10                                              20
      Ser Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu
      TCC GGT GCT TTC CCG CCA GGT CAT TTC AAA GAT CCG AAA CGT CTG
```

```
                                  30
      Tyr  Cys Lys Asn Gly Gly Phe Phe Leu Arg Ile His Pro Asp Gly
      TAC  TGC AAA AAC GGT GGT TTT TTC CTG CGT ATC CAT CCG GAT GGT
            C
           Ser
```

```
      40                                              50
      Arg Val Asp Gly Val Arg Glu Lys Ser Asp Pro His Ile Lys Leu
      CGT GTT GAT GGT GTA CGT GAG AAA TCT GAT CCG CAT ATC AAA CTG
```

```
                                  60
      Gln Leu Gln ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val
      CAG CTG CAA GCT GAA GAG CGT GGT GTA GTT TCT ATT AAA GGT GTA
```

```
      70                                              80
      Cys Ala Asn Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu
      TGT GCT AAC CGG TAC CTG GCT ATG AAA GAA GAC GGT CGT CTG CTG
       C              Kpn I
      Ser
```

```
                                  90
      Ala Ser Lys Cys Val Thr Asp Glu Cys Phe Phe Phe Glu Arg Leu
      GCT TCT AAG TGT GTT ACT GAC GAA TGT TTC TTT TTC GAA CGT CTG
                 C              C
                Ser            Ser
```

```
      100                                            110
      Glu Ser Asn Asn Tyr Asn Thr Tyr Arg Ser Arg Lys Tyr Ser Ser
      GAA TCT AAC AAC TAC AAC ACT TAC AGA TCT CGT AAA TAC TCT TCC
                                                     A
                                                     Thr
```

```
                                  120
      Trp Tyr Val Ala Leu Lys Arg Thr Gly Gln Tyr Lys Leu Gly Pro
      TGG TAT GTA GCT CTG AAA CGT ACT GGT CAG TAC AAA CTG GGT CCG
                                                             T
                                                             Ser
```

```
      130                                            140
      Lys Thr Gly Pro Gly Gln Lys Ala Ile Leu Phe Leu Pro Met Ser
      AAG ACT GGT CCG GGT CAG AAA GCT ATC CTG TTT CTG CCG ATG TCT
```

```
         147
      Ala Lys Ser End End
      GCT AAA TCT TAA TAG CTCGAGAAGCTT
                             Hind III
```

FIG.2
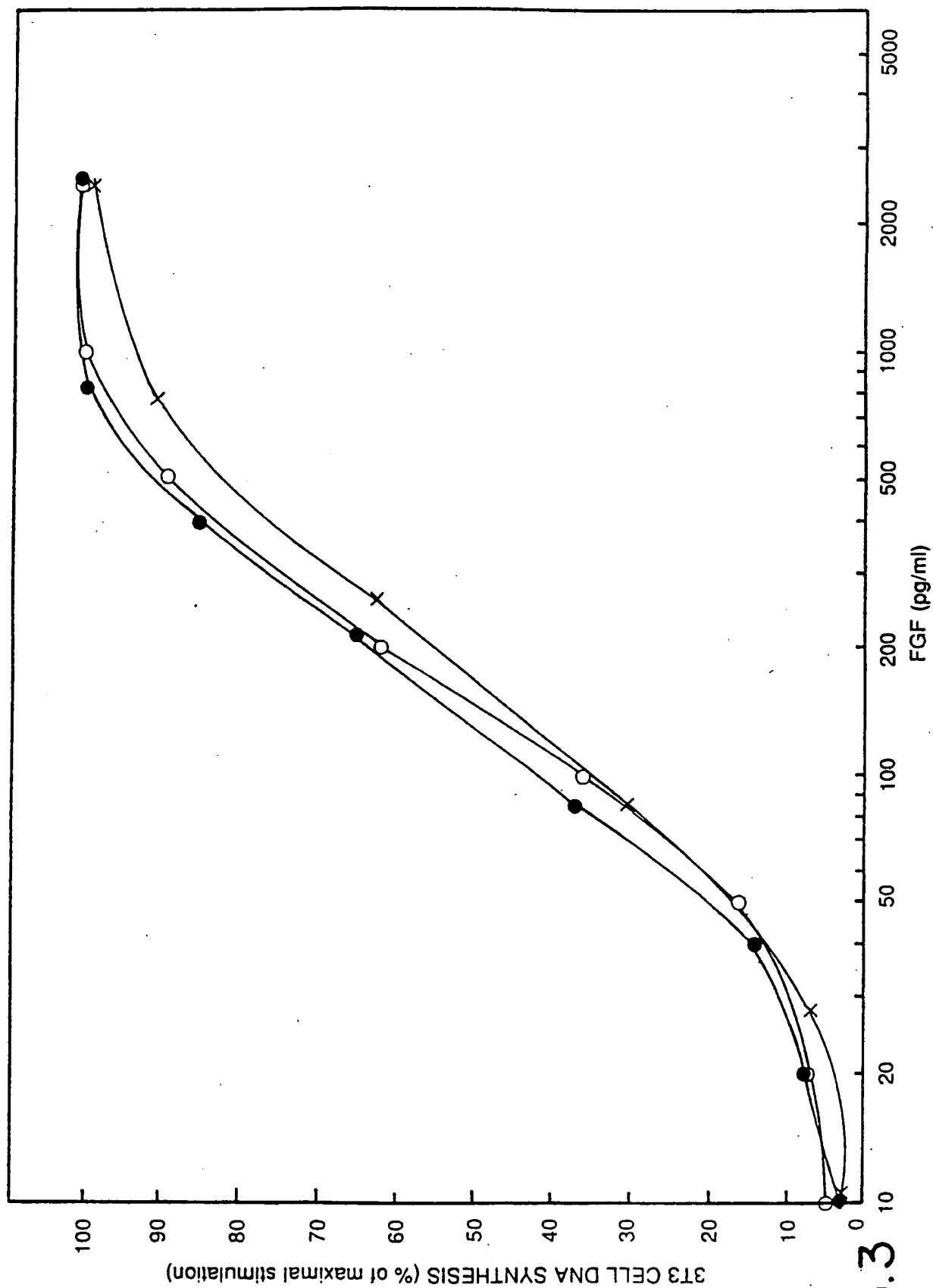
FIG.3

3T3 CELL DNA SYNTHESIS (% of maximal stimulation)

FGF (pg/ml)

FIG.4

FIG.5a
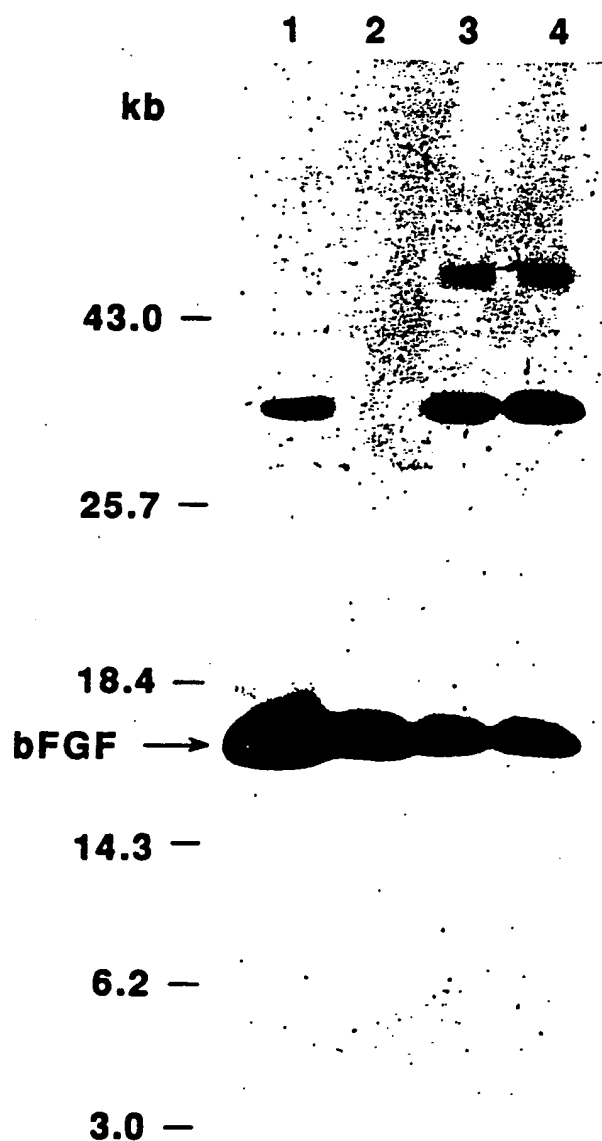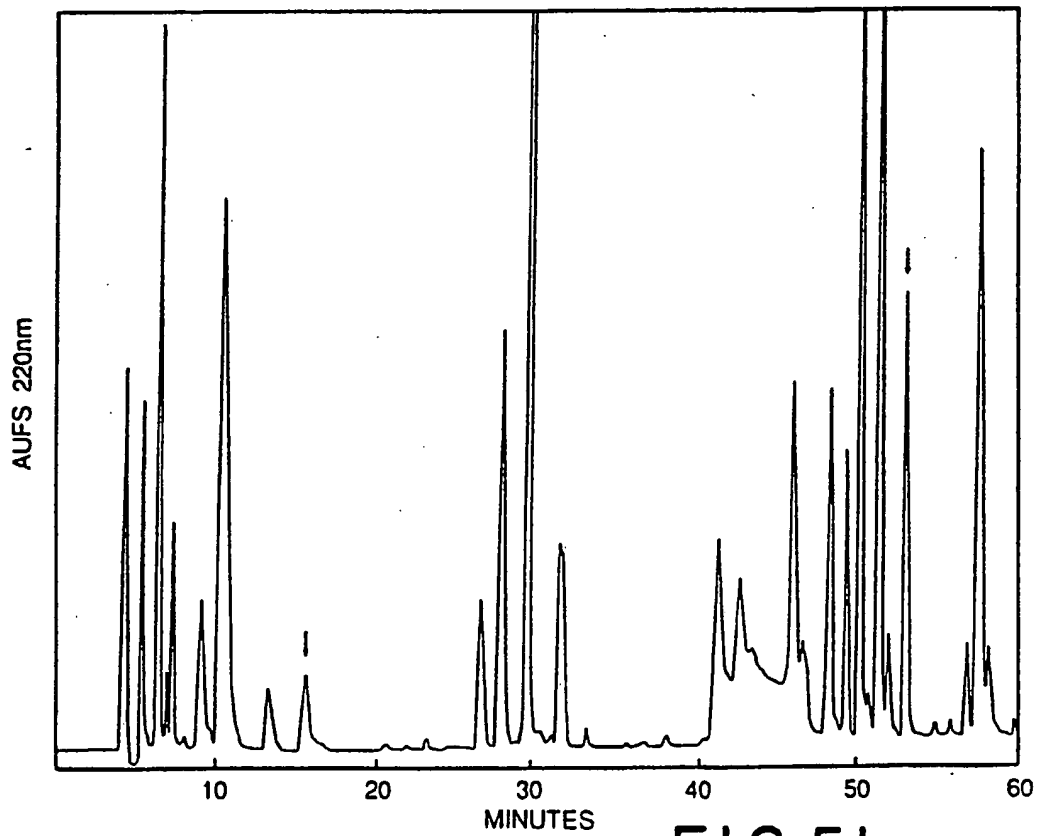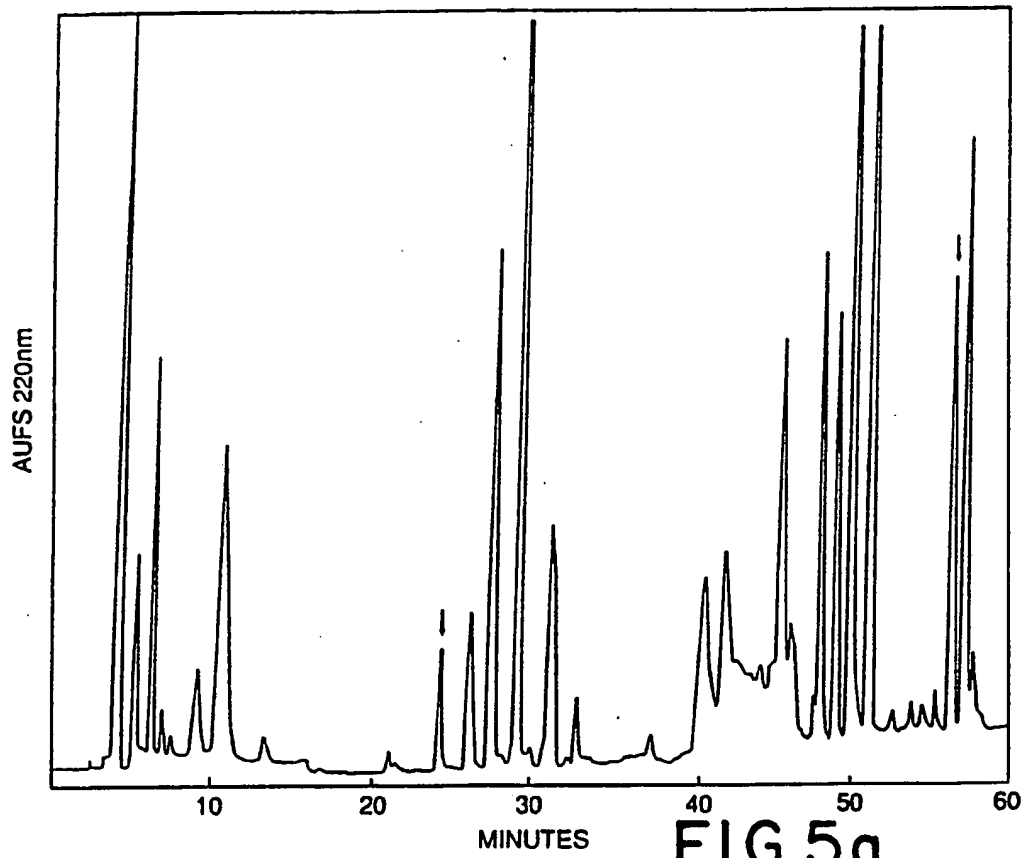
FIG.5b
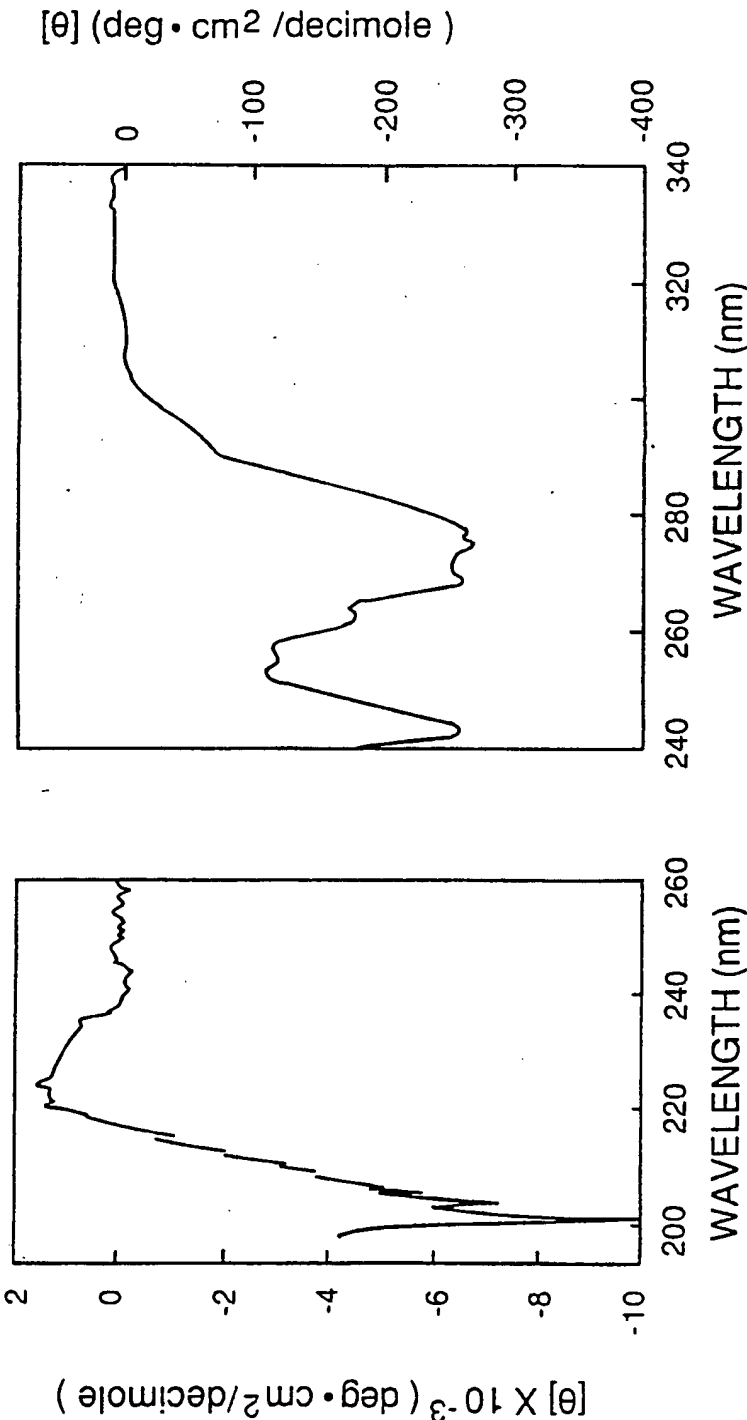
FIG.6
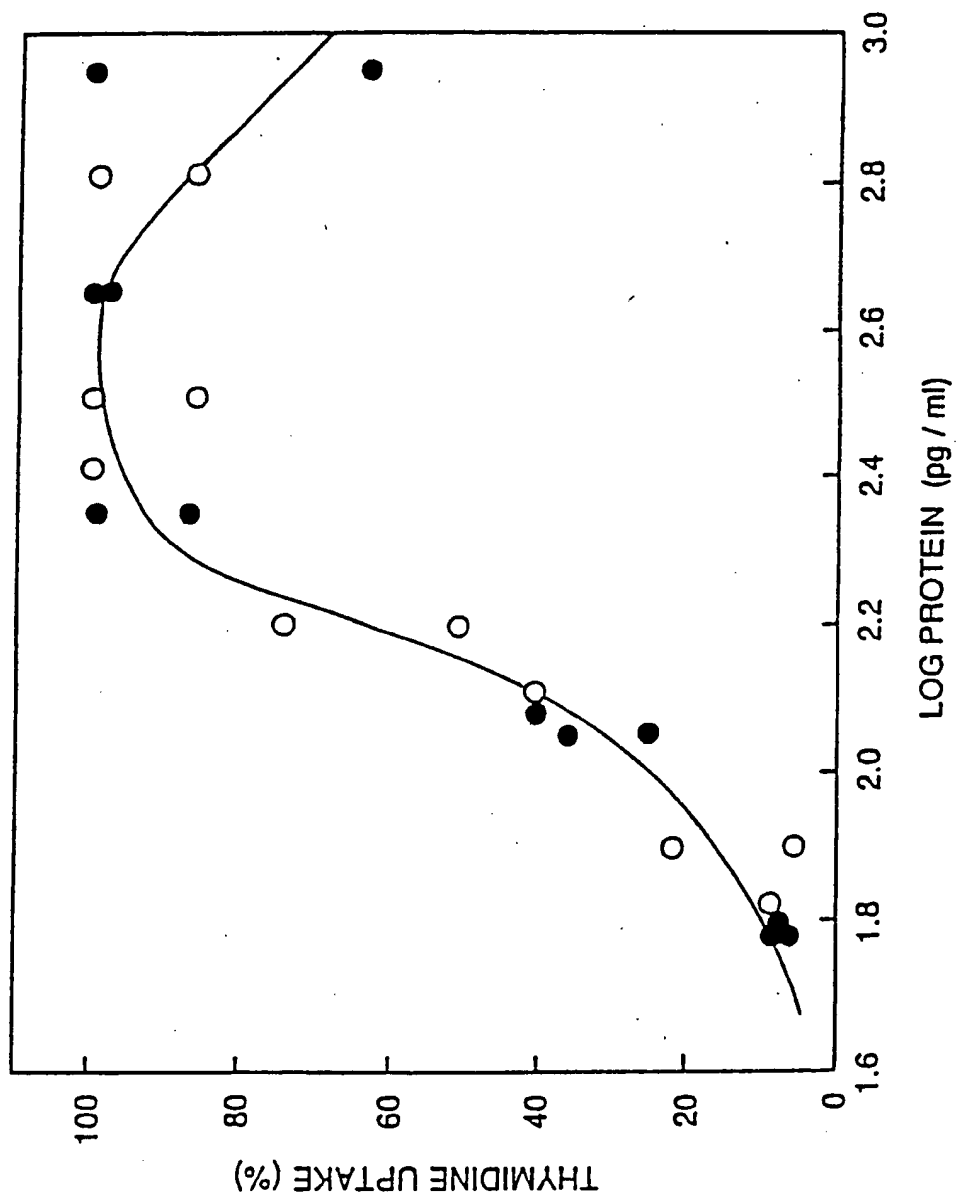
FIG.7
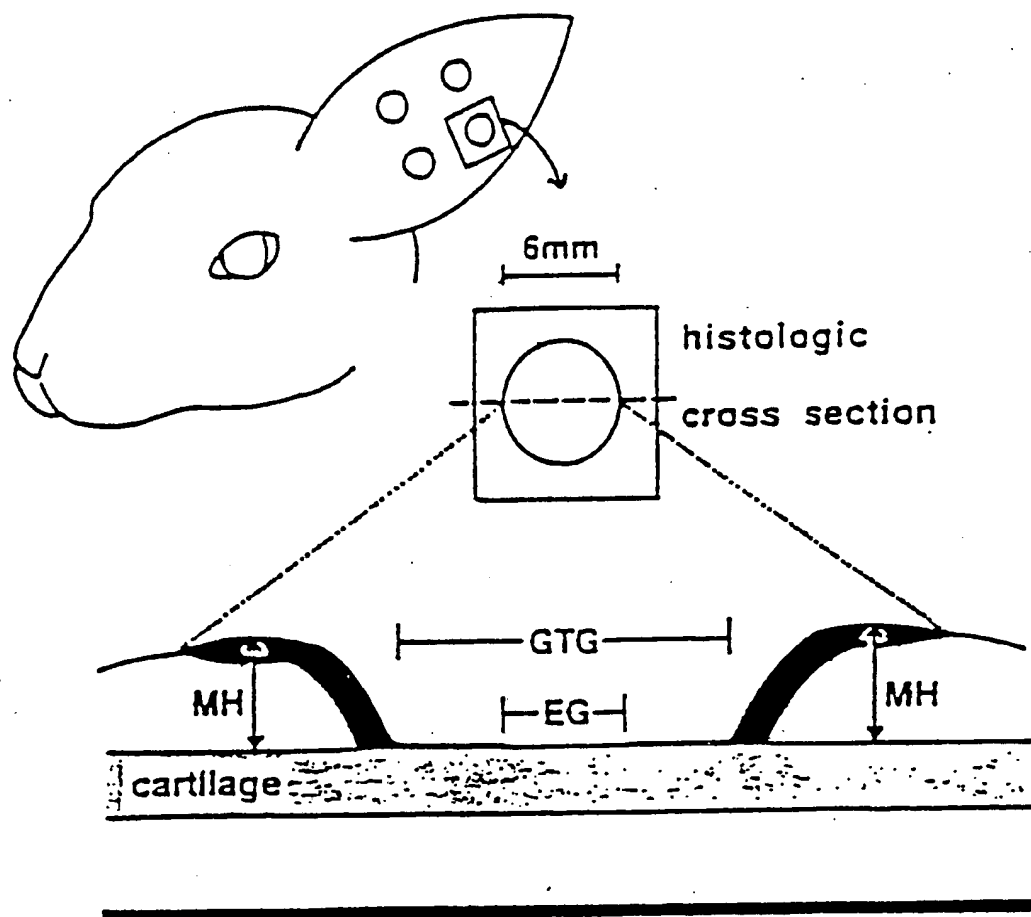
MH – maximum height of granulation tissue
GTG – granulation tissue gap
EG – epithelial gap

FIG.8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/04189

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4):C07H 15/12;C07K 13/00;C07K 3/12;A61K 37/48; see attach.
U.S. CL.: 536/27; 530/350, 412; 514/2; 435/68, 172.3 see attach.

II. FIELDS SEARCHED

Minimum Documentation Searched 7	
Classification System	Classification Symbols
U.S.	536/27; 530/350, 412; 435/68,172.3, 240.1, 253; 514/2; 935/6,9,10,11,12,13,22,23,24,59,60,61,66,67,68,69,70

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

Chemical Abstract Data Base (CAS) 1967-1989; BIOSIS DATA BASE
1969-1989; Keywords:Fibroblast,Growth,Factor,FGF,bFGF,aFGF,
see attachment.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	THE EMBO JOURNAL, Volume 5, issued 1986, September (Oxford, England), (J.A. ABRAHAM ET AL.), "Human Basic Fibroblast Growth Factor: Nucleotide Sequence and Genomic Organization" See pages 2523-2528, See particularly page 2523 and 2525.	1-31
Y	US, A, 4,296,100 (W.P. FRANCO) 20 October 1981, See abstract and columns 1-2.	21 and 27
Y	THE JOURNAL OF CELL BIOLOGY, Volume 100, issued,1985,April(J. DAVIDSON, ET AL), "Accelerated Wound Repair Cell Proliferation and Collagen Accumlation Are Produced by a Cartilage-derived Growth Factor", see pages 1219-1227, See particularly pages 1219, 1220, 1221 and 1227.	21-27

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

22 February 1989

Date of Mailing of this International Search Report

0 7 APR 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

RICHARD C. PEET

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	PCT/WO86/07595, published 31 December 1986, The Salk Institute for Biological Studies, See entire document, See particularly pages 2-7, 10-11, 17-18 and 20-22.	1-31
Y	PCT/WO87/01728, published 26 March 1987, Biotechnology Research Partners, Ltd. See entire document, See particularly pages 19-20 and 59-60.	1-31
X,P Y,P	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 151, issued 1988, March (New York, N.Y. U.S.A.) (M. SENO ET AL), "Stabilizing Basic Fibroblast Growth Factor Using Protein Engineering", See pages 701-708.	1-20 and 28-31 21-27
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES U.S.A., Volume 84, issued 1987, August (Washington,D.C., U.S.A.), (S.M. SAMSON ET AL), "Analysis of the Role of Cysteine Residues in Isopenicillin N Synthetase Activity by Site-Directed Mutagenesis", See pages 5705-5709, See particularly page 5705.	1-20
Y	BIOLOGICAL ABSTRACTS, Volume 81, no. 9, issued 1986, May, (Philadelphia, PA, U.S.A.), S. Liang et al, "Studies of Structure-Activity Relationships of Human Interleukin 2", see page 191, column 2, the abstract no. 81888, J. Biol. Chem., 1986, 261(1): 334-337 (Eng).	1-20
Y,P	CHEMICAL ABSTRACTS, Volume 108, no. 13, issued 1988, March, 28 (Columbus, Ohio, U.S.A), J.R. Winther et al. "The Free Sulfhydryl Group (Cys341) of Carboxy peptidase Y: Functional Effects of Mutational Substitution see page 298, column 1, the abstract no. 108793y, Carlsberg Res. Commun, 1987, 52 (4):263-273 (Eng).	1-20
Y	SCIENCE, Volume 219, issued 1983, January (Washington, D.C., U.S.A) (M.B. SPORN ET AL), "Polypeptide Transforming Growth Factors Isolated from Bovine Sources and Used For Wound Healing In vivo".	21-27

Attachment to PCT/ISA/210

I. Classification of Subject Matter:

IPC (4): C12P 21/00; C12N 15/00; C12N 5/00; C12N 1/20

U.S.CL.: 240.1, 253

II. Fields Searched

keywords: mutant, mutation, replace, substitution,

sulfhydryl, cysteine, disulfide, bridg?, antagonis?